

Anti-Inflammatory and antiviral effects of water-soluble crude extract from *Phragmites australis* in vitro

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Abstract: *Phragmites australis* (*P. australis*), a world wide distributed wetland grass, is traditionally used as food-making helper and spice in China. The pharmacological effect of this plant is poorly understood. Here, we demonstrated that lipopolysaccharide (LPS)-induced production of inflammatory mediators nitric oxide (NO) and reactive oxygen species (ROS), and the pro-inflammatory cytokines tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) in RAW264.7 macrophage were significantly inhibited by the crude extract. The inflammation pertinent signaling extra cellular signal-regulated kinase 1/2 (Erk1/2), P38MAPK, C-Jun and NF-kappaB (NF- κ B) activated by LPS could be dramatically inhibited by this extract. It also remarkably inhibited bovine herpes virus type 1 (BoHV-1) replication in MDBK cells. Taken together, here, for the first time we provided *P. australis* a novel natural herb as a potential candidate for the generation of antiviral and anti-inflammatory agent.

Keywords: *P. australis*, ROS, NO, inflammation, BoHV-1.

INTRODUCTION

Both acute and chronic inflammatory responses are associated with the recruiting and activating of phagocytic cells e.g. macrophages and neutrophils (Moldawer and Copeland, 1997, Palladino, Bahjat *et al.*, 2003). The recruited phagocytic cells produced various inflammatory mediators, such as ROS and NO, and pro-inflammatory cytokines, including IL-1 β and TNF- α (Nakagawa, Arai *et al.*, 2012, Zhang, Feng *et al.*, 2013). NO, ROS, TNF- α and IL-1 β have been reported as important factors for the etiopathogenesis in a series of diseases, e.g. in septic syndrome, rheumatoid arthritis and virus infection (Bauer and Bauer, 1999, Bauerova and Bezek, 1999, Ye, Huang *et al.*, 2013, Zhang, Feng *et al.*, 2013). Thus, the depression of both inflammatory mediators and pro-inflammatory cytokines with medication is widely employed in cases of inflammation-related diseases.

Herbs with convinced pharmacological effects providing potential resource for the generation of novel medicines with high effectiveness and very few side effects. As a flavor, the leaves of *P. australis* are conventional used for cooking in China. While, till now the pharmacological activities of the leaves of *P. australis* is rarely reported.

In order to find novel herbs for the generation of novel anti-inflammatory reagents from natural resource, we evaluated the pharmacological effect of the water-soluble

extract from leaves of *P. australis*, through analysis the anti-inflammatory and antiviral effects. And revealed that the leaf of *P. australis* maybe a promising herb for the development of novel anti-inflammatory and antiviral agent(s).

MATERIALS AND METHODS

Virus and cell cultures

RAW264.7 cells and MDBK cells were cultured in RPMI-1640 (Gibco BRL) medium with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), and DMEM medium (Gibco, BRL) containing 10% horse serum (HyClone Laboratories, Logan, UT), respectively. BoHV-1 of Colorado1 strain was used for this study (Wang, Menon *et al.*, 2003). The virus was amplified in MDBK cells without serum, and aliquots of viral stocks were titrated with TCID₅₀ and stored at -70°C.

Antibodies and reagents

Antibodies against phospho-p44/42MAPK(Erk1/2) (Thr202/Tyr204), phospho-p38MAPK (Thr180/Tyr182), phospho-c-Jun (Ser73), phospho-I κ B α (Ser32/36) and GAPDH were ordered from Cell Signaling Technology (cell signaling, Beverly, MA). 2',7'-Dichlorofluorescein diacetate (H2DCFDA), Thiazolyl Blue Tetrazolium Bromide (MTT), cocktail of protease and phosphatase inhibitors, N-Acetyl-L-cysteine (NAC) and LPS were provided by Sigma Aldrich (Sigma-Aldrich, St. Louis,

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MO). RNAiso plus kit and Prime Script™ II 1st strand cDNA Synthesis Kit were provided by TaKaRa (Dalian, China). SYBR real-time PCR premix was provided by ABGene (Rochester, NY). Enhanced chemiluminescence (ECL) Western blotting substrate was ordered from Thermo Scientific.

The preparation of water-soluble crude extracts from leaves of *P. australis*

The leaves used in this preparation were grown at the river side of Yangtze River at the range of Yangzhou district. At May 2014, sprouts of *P. australis* were collected, briskly washed with water and cut into chips. Distilled water of 500mL was added to the leaf chips of 50g. After boiling for 20min, the liquid was further concentrated over mild heating. The debris was discarded until about 50mL of liquid was remained. The concentrated extract was subjected to centrifugation at 13000 rpm for 5min. The supernatant was lyophilized to get a crude extract. Approximately, 10mg of water-soluble powder was obtained from the plant material.

Cellular toxicity analysis

The cellular toxicity was measured based on MTT assay. Monolayer of both RAW 264.7 macrophage cells and MDBK cells in 96-well plates were mock treated or treated with the water-soluble extract from the leaves of *P. australis* at various concentrations of 1, 10, 100 and 200 mg/mL, respectively. At 24 h post treatment, the cells were incubated with 0.5 mg/ml MTT solution. Cell viabilities were estimated by the determination of the optical density (OD) at wave length of 550 nm. The mean OD value of the cell control was assigned as 100%.

Assay for the intracellular ROS

Production of intracellular ROS was determined using the oxidative conversion of H2DCFDA to fluorescent compound dichlorofluoresce in (DCF). Monolayer of RAW264.7 cells in 24-well plates at a density of 5×10^5 cells/well were first pretreated with crude extract from leaves of *P. australis* at various concentrations of 1, 10, 50 and 100µg/mL for 4h at 37°C. Subsequently, the cells were stimulated with LPS at a concentration of 1µg/mL together with or without the extract for 3 h. In parallel, the treatment of cells with ROS scavenger NAC of 1mg/mL was used as a control. After extensive washing with PBS, the cells were exposed to H2DCFDA at a concentration of 50µM for 30 min at 37°C. Finally, the cells were washed twice with PBS, and images were acquired under a fluorescence microscope (Olympus BX-51).

The characterization of cellular NO generation

As an indicator of NO generation, the nitrite concentration in the cell culture supernatant was determined with Griess reagent. In brief, monolayer of RAW 264.7 cells in 24well plates (at a concentration of 5×10^5 cells/well) were mock treated or treated with crude

extract from leaves of *P. australis* at the concentrations of 1, 10, 50 and 100µg/mL for 4 h at 37°C. And followed by the stimulation with 1µg/mL of LPS in the presence of crude extract for 24h. Griess reagent Kit (Beyotime, Jiangsu, China) was applied to determine the nitrite concentration in the supernatants following the manufacture's specification. Standard nitrite solutions were employed to get a standard curve via the OD value of detected concentrations at the wavelength of 540 nm. The amount of nitrite in the samples was calculated through the standard curve.

RNA preparation and qRT-PCR analysis

RAW 264.7 cells of 1×10^6 cells/well in 6-well plates were pretreated with crude extract from leaves of *P. australis* at indicated concentrations for 4h, followed by stimulation with LPS together with or without extract for 24h. Cellular RNA was purified from each sample using RNAiso plus kit following the manufacture's instruction. And fresh RNA (1µg) was used as a template for the synthesis of the first strand cDNA with commercial oligodT primers using Prime Script™ II 1st strand cDNA Synthesis Kit following the manufacturer's instructions. The products were used as templates to relatively quantitate the gene expressions of both TNF-α and IL-1β. Real-time PCR was carried out using the ABI 7300 detection System (Applied Biosystems, CA). Primer sequences were illustrated in table 1. Gene expression of both TNF-α and IL-1β was normalized to the internal control glyceraldehyde-3-phosphate dehydrogenase (GADPH).

BoHV-1 replication assay

Monolayer of MDBK cells in 24-well plates were mock pretreated or pretreated with 1, 10 and 100µg/mL of water-soluble crude extract from leaves of *P. australis* for 1 h at 37°C, followed by infection with BoHV-1 at a multiplicity of infection (MOI) of 1. After treatment with citric buffer and extensive washing with PBS, DMEM medium (400µL) with or without crude extract was added to each well. At 24 hpi, the cell cultures were subjected to repeated freeze-thawing cycles, and the progeny virus was determined by TCID₅₀ assay.

To determined at which stage the virus replication was affected by the extract, monolayer of MDBK cells in 24 well plates were treated with extract from leaves of *P. australis* at various stages including throughout infection, before virus inoculation for 1h and during the virus inoculation, and after virus inoculation for 1h. After 48 h of infection, the virus was tittered by TCID₅₀ assay.

Western blotting

RAW 264.7 cells of confluent in 60-mm dishes were mock treated or pretreated with crude extract from leaves of *P. australis* at the concentrations of 1, 10 and 100µg/mL for 4 h, followed by the stimulation with LPS

Table 1: Primers used for the real-time PCR

Primer specificity	Forward primer	Reverse primer
TNF- α	5'-TGCCTATGTCTCAGCCTCTTC-3'	5'-GAGGCCATTTGGGAACCTT CT-3'
IL-1 β	5'-TGTAATGAAAGACGGCAC ACC-3'	5'-TCTTCTTTGGGTATTGCT TGG-3'
GAPDH	5'-TATGTCGTGGAGTCTACTGGT-3'	5'-GAGTTGTCATATTTCTCGTGG-3'

in the present or absent of the extract for 10 min. Cells were washed twice with ice cold PBS and lysed in radio immunoprecipitation assay buffer as described elsewhere (Zhu, Yang *et al.*, 2013). The cell debris was pelleted by centrifugation at 13,000 rpm for 10 min at 4°C. The cell lysates were separated by 10% SDS-PAGE and transferred to a nitrocellulose membranes. The membranes were blocked in 5% dry milk in TBST for 1h at room temperature, followed by the incubation with primary antibodies over night at 4°C, and subsequent HRP conjunct secondary antibody for 1h at room temperature. The immuno reactive bands were visualized by chemiluminescence method.

RESULTS

To measure the toxicity of water-soluble extract from leaves of *P. australis* to the cell lineages used for this investigation, cell viability of both RAW 264.7 macrophages and MDBK cells exposed to the extract at different concentrations were evaluated. Under these conditions, the extract showed no cytotoxicity except at a concentration of 200 μ g/mL, which leading to a 8.81% and 12.03% decrease in cell viability for RAW 264.7 macrophages and MDBK cells, respectively (fig.1). Thus a maximum concentration of 100 μ g/mL of the extract was used for the subsequently research.

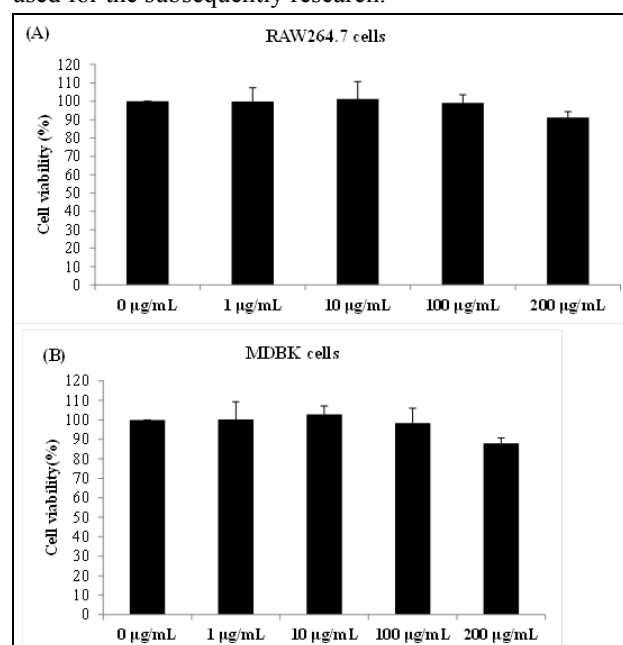


Fig. 1: Effects of water-soluble crude extract from leaves of *P. Australis* on the viability of both RAW 264.7 macrophages and MDBK cells. Cells were treated with

indicated concentrations (0, 1, 10, 100 and 200 μ g/mL) of the extract for 24h prior to cell viability assessment with MTT reduction assay. The results are expressed as the percentage of surviving cells over control cells. Each value indicates the mean $\pm$ SD and the error bars indicate the standard deviations of two independent experiments.

Water-soluble crude extract from leaves of P. Australis inhibited LPS-stimulated ROS and NO generation in RAW264.7 macrophages

LPS is one of the most potent stimulators for both ROS and NO generation, *in vitro*. Here, we used this model to estimate the antioxidant activity of water-soluble crude extract from leaves of *P. Australis*. With this system, ROS was obviously induced at 4 h post treatment with LPS in macrophages. Similarly to the treatment with ROS scavenger NAC, the crude extract at all the detected concentrations completely depressed LPS-induced ROS (fig. 2A). As demonstrated in fig. 2B, LPS stimulation markedly elevated NO production to a concentration of 40.16 $\pm$ 0.19 μ M after treatment for 24h. While, the elevated production of NO was significantly damped by the treatment with crude extract with a dose dependent manner. Treatment of the cells with the extract at a concentration of 100 and 50 μ g/mL resulted in the generation of NO to a low level of 5.19 $\pm$ 0.14 μ M and 15.48 $\pm$ 2.37 μ M, respectively. Collectively, it evidenced that some component(s) in the crude extract from leaves of *P. Australis* possessed strong antioxidant effect.

Water-soluble extract from leaves of P. Australis inhibited LPS-induced expression of IL-1 β and TNF- α

Pro-inflammatory cytokines IL-1 β and TNF- α , primarily secreted by macrophages are important biomarker of inflammation. Here, with qRT-PCR we examined the effect of the extract on the expression of IL-1 β and TNF- α in RAW264.7 macrophages. As a result, the crude extract was evidenced to inhibit LPS-induced transcription of both TNF- α and IL-1 β with varied capacity (fig. 3). Only at a higher concentration of 100 μ g/mL the extract could suppress TNF- α transcription (fig. 3A). While IL-1 β was inhibited by the extract at a concentration of both 100 and 10 μ g/mL (fig. 3B). Collectively, these data evidenced that some component (s) in the crude extract from leaves of *P. Australis* possessed strong anti-inflammatory activity.

Extract from leaves of P. Australis Affected NF- κ B and MAPK Signaling

Since both NF- κ B and MAPKs signaling including the Erk1/2, p38 MAPK and C-Jun are inflammation pertinent

pathways. Here, the regulation of these signaling by the crude extract in RAW 264.7 cells was investigated. As demonstrated by fig. 4 the phosphorylation of p38MAPK, C-Jun, Erk1/2 and I κ B α caused by the stimulation with LPS was broadly ameliorated by the crude extract at various concentrations indicated. These results suggest that some component(s) in the crude extract from *P. Australis* possessed anti-inflammatory effect via affecting the inflammation related signaling, such as NF- κ B and MAPK signaling cascades.

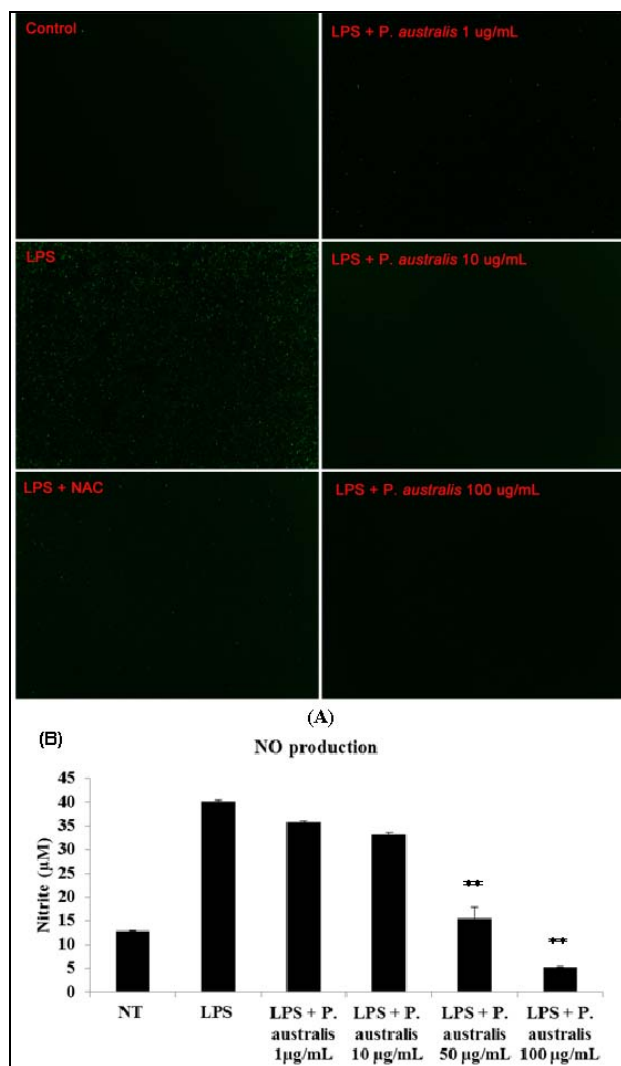


Fig. 2: Effects of water-soluble crude extract from leaves of *P. Australis* on the generation of ROS and NO stimulated by LPS in RAW264.7 macrophages. RAW 264.7 macrophages in 24-well plates were pretreated with or without the extract at indicated concentrations for 4h. The cells were then stimulated with 1 μ g/mL of LPS with or without crude extract for 4 h, prior to loading with H2DCFDA (50 μ M, 30min) for visualization of ROS using fluorescence microscopy (A). The data is one present of three experiments. Or the cells were treated with 1 μ g/mL of LPS for 24 h, the amount of NO in the cell culture supernatants was measured by Griess reaction

assay (B). The data represent mean $\pm$ SD of triplicate experiments. The data was analyzed with student *t* test. ** p <0.01.

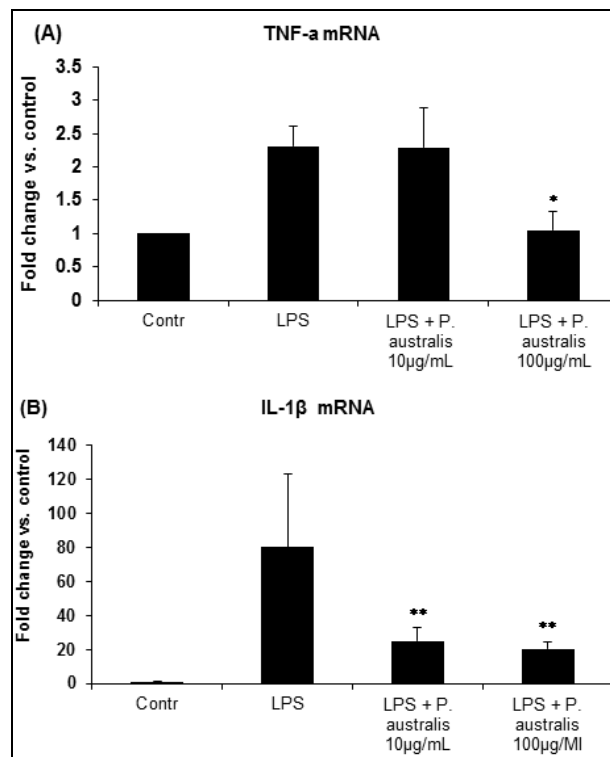


Fig. 3: Water-soluble crude extract from leaves of *P. Australis* inhibited the expression of TNF- α and IL-1 β in RAW264.7 macrophages. RAW264.7 macrophages were pretreated with the extract at indicated concentrations for 4 h, followed by the stimulation with LPS (1 μ g/mL) for 24 h. Total RNA was extracted and mRNA of TNF- α and IL-1 β was analyzed using qRT-PCR. The data was analyzed with student *t* test, * p <0.05 and ** p <0.01

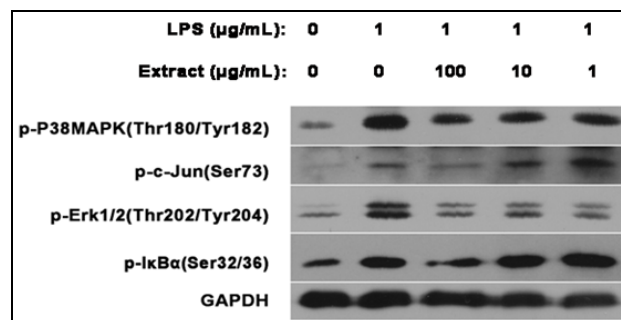


Fig. 4: Effects of water soluble crude extract of *P. Australis* on LPS-induced activation of MAPK and NF- κ B signaling. RAW264.7 cells were incubated with or without the crude extract at indicated concentrations for 4 h, then stimulated with LPS (1 μ g/mL) with or without of the extract for 10min. Cytosolic lysates were separated by SDS-PAGE and transferred to PVDF membrane. Subsequently, the proteins of phospho-P38MAPK, phospho-C-Jun, phospho-ERK1/2, phospho-I κ B α and GAPDH were detected by Western blotting. Data

presented one of three independent experiments.

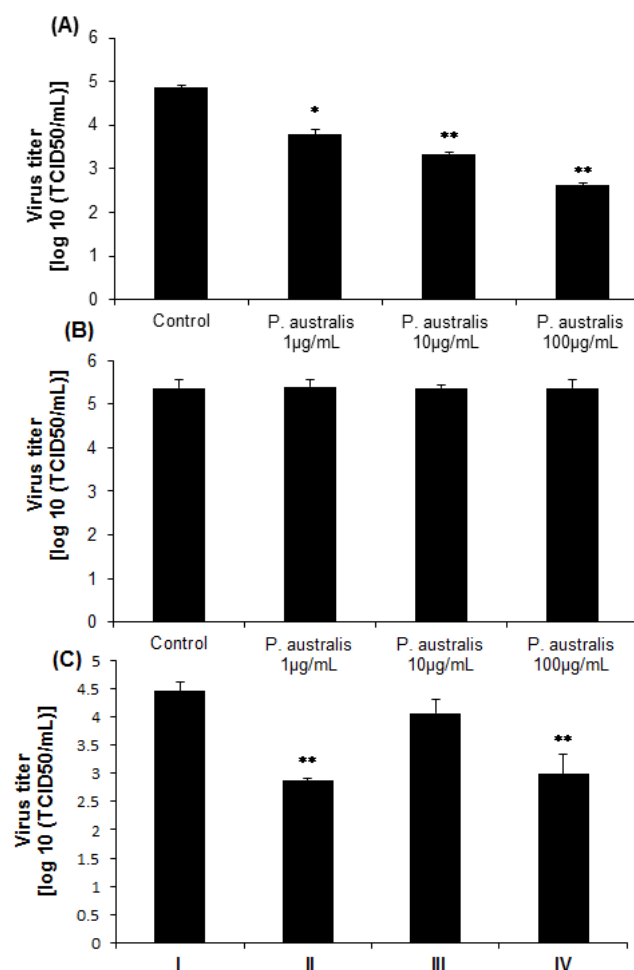


Fig. 5. Antiviral activity of water-soluble crude extract of *P. Australis* on BoHV-1 infection in MDBK cells. MDBK cells infected with BoHV-1 at an MOI of 1 were treated with the extract at indicated concentrations throughout infection plus a pretreatment for 1 h prior to infection (A). The virus incubated with the extract at indicated concentrations for 2 h at 37°C, were titrated with TCID₅₀ assay (B). Monolayers of MDBK cells infected with BoHV-1 at MOI 1 were treated with 100μg/mL of crude extract at different phases of infection: (I) mock treatment control (II) treatment throughout infection plus a pretreatment for 1h, (III) treatment during the virus inoculation for 1h and plus a pretreatment for 1h, (IV) treatment after virus inoculation for 1h until 24 hpi. After 24 h of infection, the virus was titrated with TCID₅₀ assay (C). The assay were performed in duplicate and data represent means $\pm$ SD, The data was analyzed with student *t* test. ** <0.01 .

Crude extract from *P. Australis* affected BoHV-1 infection in MDBK cells

BoHV-1 replication in MDBK cells was employed to assess the antiviral activity of the crude extract from *P. Australis*. As showed in fig. 5a, the water-soluble crude

extract impaired the virus propagation with a dose-dependent manner. As a 2.2, 1.5 and 1.2-log of virus yield was reduced by the treatment of the cells with the extract at the concentrations of 100, 10 and 1μg/mL, respectively.

Since, the treatment of the virus with the extract could not inactivate the viral particles (fig. 5B), the inhibitory effect of the extract was not due to the virucidal effect of the extract, but affecting certain cellular signaling which is crucial for the virus infection process. Thus, at which stage (s) the virus replication was affected by the extract was further investigated. As showed in fig. 5C, the extract mainly affected the virus post-entry stage.

DISCUSSION

Diverse pathogens, e.g., influenza A virus, triggers “cytokine storms”, involving over production of pro-inflammatory cytokines, such as IL-1 β , TNF- α , IL-8 and IL-6 (D’Elia, Harrison *et al.*, 2013, Tisoncik, Korth *et al.*, 2012). Multiple studies indicated that excessive production of inflammatory mediators ROS and NO is detrimental to the body, and responsible for the oxidative stress (Gerlach, Keh *et al.*, 1998, Juurlink and Paterson, 1998). Besides targeting the pathogens, to suppress the production of both pro-inflammatory cytokines and ROS is emerging acknowledged as a powerful therapeutic strategies for the treatment of a case with server inflammation (D’Elia, Harrison *et al.*, 2013, Vlahos, Stambas *et al.*, 2012).

To develop a medicine with combined effect against both pro-inflammatory cytokines and ROS from natural resources, such as herbs with known pharmacological effect may be an effective approach. The exact pharmacological effect of water-soluble extract from leaves of *P. Australis* is rarely reported. Here, for the first time we reported that the extract was a potential candidate for the generation of medicine with strong antioxidant, anti-inflammatory and antiviral effects.

In this study, the antioxidant activity of the extract was certified by the fact that it could significantly block LPS-induced production of intracellular NO and ROS in RAW264.7 cells, meanwhile anti-inflammatory activity was assessed by inhibition of IL-1 β and TNF- α expression (fig 2 and 3). Generally, the transcription of the cytokines were regulated by signaling, such as NF- κ B, Erk1/2, C-Jun and p38 MAPK pathways. Therefore, We assessed whether this extract also affected these signaling in response of LPS stimulation. The results indicated that the crude extract blunted the activation of the indicated signaling (fig. 4). Taken together, the water-soluble crude extract from reed leaves has broad effect on the depression of inflammation pertinent signaling.

Interestingly, we found that the water-soluble crude extract from leaves of *P. Australis* also exert strong

antiviral effect as identified by suppressing BoHV-1 replication in MDBK cells at the post-entry stages. However, whether it possessed broad antiviral effect need further identification. To our knowledge, this is the first reported herb or plant that could inhibit BoHV-1 replication. Especially, the leaves of *P. Australis* can be feed for the cattle. So it would be beneficial for the healthy of the cattle.

In summary, for the first time our study indicated that the crude extract from leaves of *P. Australis* has potent antioxidant, anti-inflammatory and antiviral activities. Though the component (s) in the crude extract responsible for the pharmacological effects is unclear at the moment. We provide a valuable natural resource, and a simple preparation method for the generation of novel antiviral and anti-inflammatory agent.

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