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Forsythiae Fructus attenuates cisplatin-induced cytotoxicity in IEC-6 cells and J774A.1 macrophages by inhibiting NLRP3/caspase-1/GSDMD mediated pyroptosis

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ABSTRACT

Objective: Forsythiae Fructus (lian qiao in Chinese), the dried fruit of *Forsythia suspensa* (Thunb.) Vahl, is a commonly used traditional Chinese medicine known for its diverse biological activities, including antiemetic, anti-inflammatory, antioxidant, antiviral, and neuroprotective properties. This study investigated the protective effects of Forsythiae Fructus and its primary components, phillyrin and forsythoside A, against cisplatin-induced cytotoxicity *in vitro*, specifically focusing on the intestinal epithelial cells (IEC-6) and the J774A.1 macrophage cell line.

Methods: Cisplatin and *tert*-butyl hydroperoxide (tBHP) were used to induce stress in IEC-6 cells, while cisplatin and lipopolysaccharides (LPS)/adenosine triphosphate (ATP) were employed for J774A.1 macrophages. The protective effects of Forsythiae Fructus aqueous extract (FAE), phillyrin, and forsythoside A against cytotoxicity in these cultured cells were evaluated. Cell viability was assessed using the Cell Counting Kit-8 assay, while cell membrane permeability was determined through Hoechst 33342 and propidium iodide staining. Intracellular reactive oxygen species (ROS) levels were investigated using DCFH-DA, and the expression of mRNA and protein related to the NLRP3 inflammasome and GSDMD-induced pyroptosis was quantified through qRT-PCR and western blotting.

Results: In IEC-6 cells, combining FAE, phillyrin, or forsythrin A with a subthreshold dose of the antioxidant N-acetyl-L-cysteine (NAC) significantly mitigated cisplatin- or tBHP-induced cell necrosis and restored impaired cell viability. Additionally, the upregulation of NF-κB, ASC, NLRP3, caspase-1, GSDMD, and HMGB1 at both mRNA and protein levels induced by cisplatin or tBHP was markedly reversed with the joint intervention of FAE, phillyrin, or forsythrin A with NAC. Similarly, in cisplatin- or LPS/ATP-treated J774A.1 macrophages, the effects on cell necrosis, cell viability, and the NLRP3/caspase-1/GSDMD pathway mirrored our previous findings in IEC-6 cells.

Conclusion: The study suggests that the alleviating effect of Forsythiae Fructus and its primary components, phillyrin and forsythoside A, against cisplatin-induced cytotoxicity may be attributed to inhibiting oxidative stress, downregulating the NLRP3/caspase-1/GSDMD pathway, and inhibiting pyroptosis.

1. Introduction

Chemotherapy-induced nausea and vomiting (CINV) are among the most prevalent and distressing side effects faced by patients undergoing antineoplastic treatments.[1] This condition can lead to complications such as dehydration,[2] metabolic imbalances,[3] anorexia,[4] and weakened physical stamina. Notably, highly emetic drugs such as cisplatin are notorious for causing severe nausea and vomiting in approximately 90% of patients.[5] Therefore, enhancing the management of CINV is of utmost importance. CINV is closely linked to the release of various neurotransmitters, including 5-hydroxytryptamine (5-HT) and substance P. Accordingly, current clinical approaches to treating CINV primarily involve the use of 5-HT_3 receptor antagonists (5HT_3RAs) and NK-1 receptor antagonists (NK-1RAs).[1] However, despite these efforts, preventing CINV remains mainly unsatisfactory. As a result, there is an urgent need for innovative and efficacious therapeutic agents to against CINV.

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Chemotherapy-induced inflammation in the gastrointestinal tract is considered one of the potential pathological mechanisms of CINV.[6] Cisplatin, in particular, can lead to various structural alterations in the small intestine, including the shortening of intestinal villi, depletion of intestinal crypt infiltration of inflammation in the mucosa and submucosa layers, destruction of the gastrointestinal mucosa integrity, and impairment of the intestinal barrier function.[7,8] Increasing evidence suggests that escalating inflammatory responses and excessive reactive oxygen species (ROS) production are the primary pathological processes driving cisplatin-induced gastrointestinal inflammation.[6] Research has confirmed that cisplatin-induced gastrointestinal toxicity is mediated by activating the NOD-like receptor, pyrin domain-containing-3 (NLRP3) inflammasome, which is pivotal in regulating gastrointestinal diseases.[9,10] The NLRP3 inflammasome is an intricate intracellular complex comprising an NLRP3 sensor, the apoptosis-associated speck-like protein containing a CARD (ASC) adaptor molecule, and the procaspase-1 cysteine protease.[11] Once the NLRP3 inflammasome is triggered, it initiates the self-cleavage and activation of procaspase-1, leading to the maturation of the proinflammatory cytokines such as interleukin 1β (IL-1β) and interleukin 18 (IL-18).[12] In addition, activated caspase-1 also cleaves gasdermin D (GSDMD) and releases its N-terminal domain, further promoting a type of cell death called pyroptosis.[13]

Forsythiae Fructus, known as "Lian qiao" in Chinese, the dried fruit of *Forsythia suspensa* (Thunb.) Vahl, was first recorded in *Shennong's Classic of Materia Medica*, and it is currently recognized in the pharmacopeias of China, Japan, and Korea. Interestingly, its antiemetic properties were first recorded in Japanese Kampo literature, *Imperial Chinese Medicine*, in 1927, despite not being initially documented in traditional Chinese medicine literature.[14] Both clinical applications and preclinical studies have provided evidence of Forsythiae Fructus' antiemetic capabilities. Clinical practice has confirmed its effectiveness in reducing nausea and vomiting.[15,16] Additionally, preclinical research has shown its ability to combat emesis induced by various agents, such as digitalis in pigeons, apomorphine in dogs,[17] and apomorphine, copper sulfate, and cisplatin in minks,[18] as well as cisplatin-induced pica in rats.[10,19]

Forsythrin A and phillyrin are the key biomarker components in Forsythiae Fructus. As per the quality standard outlined in the Pharmacopoeia of the People's Republic of China (2020 edition), Forsythiae Fructus is expected to contain a minimum of 0.15% and 0.25% of forsythoside A and phillyrin, respectively. In addition to their recognized anti-inflammatory and antioxidative[20,21] properties, recent research revealed that phillyrin has anti-nausea properties.[22]

We previously reported that one of the potential mechanisms underlying the therapeutic effects of Forsythiae Fructus against CINV involved inhibiting the activation of the NLRP3 inflammasome. This was demonstrated in a rat model of cisplatin-induced pica.[10] To gain a deeper understanding of its antiemetic mechanism, particularly its anti-inflammatory properties, this study explores the protective effects of Forsythiae Fructus and its constituents, phillyrin and forsythoside A. We aim to understand how they counteract cisplatin-induced cytotoxicity by regulating the NLRP3/caspase-1/GSDMD pathway-mediated pyroptosis. We employed *in vitro* models using IEC-6 cells and J774A.1 macrophages to simulate the conditions. To establish a model of small intestinal epithelial cell injury induced by cisplatin, we utilized IEC-6 cells. Additionally, we created an oxidative stress model using tBHP as a control to mimic the damage observed with cisplatin in IEC-6 cells. We also introduced a cisplatin-induced J774A.1 macrophage injury model to investigate the impact of inflammation on IEC cells. As controls, we used tBHP and LPS/ATP costimulation in the IEC-6 and macrophage damage models, respectively, to explore the roles of oxidative stress and inflammation in cisplatin-induced cytotoxicity.

2. Materials and methods

2.1. Chemicals and reagents

Forsythiae Fructus was procured from the Chinese Medicine Material Processing Plant (Foshan, Guangdong, China) and authenticated by Professor Jizhu Liu. A voucher specimen (number F5458) has been securely stored at the School of Chinese Materia Medica, Guangdong Pharmaceutical University. Forsythoside A and phillyrin were acquired from Chengdu Master Biotechnology Co., Ltd. (Chengdu, China), while the authentic standard chemicals were sourced from the National Institutes for Food and Drug Control (Guangzhou, Guangdong, China). Cisplatin was obtained from Dalian Meilun Biotechnology Co., Ltd. (Dalian, Liaoning, China). ATP was procured from Aladdin. N-acetyl-L-cysteine (NAC), lipopolysaccharide (LPS), and *tert*-butyl hydroperoxide (tBHP) were purchased from Sigma (St. Louis, MO, USA).

2.2. Preparation of Forsythiae Fructus aqueous extract (FAE)

FAE was prepared following our previous protocol.[10] In brief, 250 g of accurately weighed Forsythiae Fructus raw material was soaked in distilled water at a tenfold ratio overnight. It was then subjected to two rounds of boiling, the first for 1.5 h and the second for 1 h. The resulting extracts were concentrated to 1000 mL under reduced pressure. Subsequently, the solution was lyophilized to yield a fine powder (72.0 g).

2.3. High-performance liquid chromatography analysis

Forsythoside A and phillyrin content in FAE was determined using high-performance liquid chromatography with an analytical column (Eclipse XD8-C18, 4.6 mm × 250 mm, 5 mm). For forsythoside A: The mobile phase consisted of acetonitrile and pure water in a 25–75 (v/v) ratio, with a flow rate of 1.0 mL/min, column temperature at 25 °C, UV detection at 277 nm, and an injection volume of 10 μL. For phillyrin: The mobile phase was composed of acetonitrile and 0.4% acetic acid in a 15–85 (v/v) ratio, with a flow rate of 1.0 mL/min, column temperature was set at 30 °C, UV detection at 330 nm, and an injection volume of 10 μL.

2.4. Cell culture and treatment

The rat intestinal epithelial cell line 6 (IEC-6) cells were kindly provided by the Institute of Spleen and Stomach at Guangzhou University of Chinese Medicine, while the mouse J774A.1 macrophages were procured from the Institute of Basic Medicine, Chinese Academy of Medical Sciences. Both cell lines were cultured in DMEM (Thermo Fisher Scientific, Beijing, China) supplemented with 10% FBS (Gibco, Carlsbad, CA, USA) and maintained in an incubator at 37 °C with 5% CO_2.

2.5. Cell viability assay

Prior to treatment, IEC-6 cells and J774A.1 macrophages were cultured in 96-well cell culture plates for 24 h. Subsequently, they were exposed to varying concentrations of the test drugs for 24 h to establish the appropriate modeling and administration concentrations. This treatment was conducted with or without forsythia fructus, phillyrin, and forsythoside A. In the case of IEC-6, they were pretreated with or without forsythia, phillyrin, and forsythoside A 1 h before being exposed to cisplatin or tBHP. Cell viability assays were then performed after 24 h of incubation. Similarly, for J774A macrophages, the cells were treated with the aforementioned drugs 1 h prior to exposure to cisplatin or LPS.

The cells were categorized into nine groups: (I) control group (DMEM medium), (II) model group (cisplatin/tBHP/LPS-ATP), (III) NAC group (NAC + cisplatin/tBHP/LPS-ATP), (IV) FAE group (FAE + cisplatin/tBHP/LPS-ATP), (V) ph group (phillyrin + cisplatin/tBHP/LPS-ATP), (VI) FA group (forsythoside A + cisplatin/tBHP/LPS-ATP), (VII) FAE + NAC group (FAE + NAC + cisplatin/tBHP/LPS-ATP), (VIII) ph + NAC group (phillyrin + NAC + cisplatin/tBHP/LPS-ATP), and (IX) FA + NAC group (forsythoside A + NAC + cisplatin/tBHP/LPS-ATP). Cell viability was assessed by employing the Cell Counting Kit-8(CCK-8) (SA613, Dojindo Japan). In brief, the treated cell medium was substituted with 10 μL of CCK-8, followed by incubation at 37 °C for 4 h. The optical density was measured at 450 nm using a microplate reader (SpectraMax ®i3x, Molecular Devices, USA). Notably, when LPS was introduced for a 24-h incubation, ATP was subsequently added, and cell viability was measured 30 min later.

2.6. Hoechst 33342/propidium iodide (PI) staining

Cell morphology was assessed using double staining with the fluorochromes Hoechst 33342 and PI (Beyotime). Following the manufacturer's instructions, dye and cell staining buffers were added to each well and incubated at 4 °C for 30 min in a dark environment. Subsequently, the cells are observed under a fluorescence microscope (CX31, Olympus, Tokyo, Japan).

2.7. Assessment of ROS

Cells were seeded in 6-well plates at a density of 1×10^5 cells/mL and incubated for 24 h. The test drugs were administered as a pretreatment for 1 h, followed by incubation with cisplatin, tBHP, or LPS/ATP for an additional 24 h. The cells were washed with PBS three times and then treated with 10 μM DCFH-DA (Beyotime) at 37 °C for 20 min in the absence of light. After this, the cells were washed again with PBS and examined using a fluorescence microscope (CX31, Olympus, Tokyo, Japan).

2.8. Western blotting analysis

All samples were homogenized using RIPA lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China), and protein lysates were quantified using a BCA protein assay kit (Beyotime). Equal amounts of proteins were separated and then subjected to SDS-PAGE before being transferred to polyvinylidene difluoride membranes. These membranes were blocked with 5% skimmed milk for 1 h and subsequently probed with the following primary antibodies overnight at 4 °C: phospho–NF–κB (#3033), NF-κB (#8242), HMGB1(#6893), and IL-1β (#12703) were purchased from Cell Signaling, NLRP3(Ab214185) and IL-1β (Abcam) were obtained from Abcam, ASC (sc-514414) was gained from Santa Cruz Biotechnology, GSDMD (20770-1AP) and caspase-1/p20/p10 were purchased from (22915-1-AP) Proteintech, GAPDH (AG019) was obtain from Beyotime. Subsequently, the membranes were probed with HRP-conjugated secondary antibodies (A0208, A0216, beyotime) for 1 h at room temperature. Protein bands were analyzed using Image J software (NIH, Bethesda, MD, USA).

2.9. RNA isolation and quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted from the cells using an RNAeasy kit (Beyotime). The qRT-PCR assay was employed to analyze the mRNA expression levels of NLRP3, ASC, Caspase1, IL-18, Il-1β, GSDMD, and HMGB1. The primer sequences can be found in Supplementary Table 1. The relative gene expression was determined using the $2^{-\Delta\Delta ct}$ method, with normalization to GAPDH.

2.10. Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.0. For the cell group curve, a two-way analysis of variance (ANOVA) with Tukey's post hoc test was used. For other data, one-way ANOVA with Tukey's post hoc test was applied. All experiments were repeated at least three times, and the data were presented as mean ± SEM. A significance level of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Characterization of components in Forsythiae Fructus

According to the detection method outlined in the Chinese Pharmacopoeia, the contents of phillyrin and forsythoside A in FAE was determined to be 0.28% and 2.62%, respectively. These results can be referenced in our previous publication.[10] The chemical structures of phillyrin and forsythoside A can be found in Fig. 1.

3.2. Effect of Forsythiae Fructus on cisplatin or tBHP-induced cytotoxicity in IEC-6 cells

3.2.1. The effect of Forsythiae Fructus on cell viability of cisplatin or tBHP-stimulated IEC-6 cells

To assess the potential antiproliferative effects of cisplatin, tBHP, FAE, phillyrin, and forsythoside An under our experimental conditions, IEC-6 cells were treated with test drugs for 24 h. The results showed that FAE (1×10^{-2} g/L), phillyrin (10 μM), and forsythoside A (10 μM) did not exhibit significant antiproliferative activity on IEC-6 cells (Supplementary Figs. 1A–1C). Furthermore, the cell viability with cisplatin (20 μM) or tBHP (50 μM) treatment was approximately 50% (Supplementary Figs. 1D and 1E). Next, we examined the effect of pretreatment with FAE, phillyrin, and forsythoside A on IEC-6 cells 1 h before exposure to cisplatin or tBHP. The results revealed that FAE, phillyrin, and forsythoside A did not significantly affect cell viability. In comparison, NAC, a commonly used antioxidant, was tested for its maximum non-cytotoxicity concentration on IEC-6 cells, which was determined to be up to 5 mM using the CCK-8 assay. However, pretreatment of IEC-6 cells with 1 mM NAC before cisplatin exposure resulted in little change in cell viability (Supplementary Figs. 1F–1H).

Hereafter, we explored the combined use of FAE, phillyrin, and forsythoside A with a subthreshold dose of NAC for cell pretreatment, as illustrated in Fig. 2A. Notably, when FAE, phillyrin, and forsythoside A were combined with 1 μM NAC, cell viability was significantly improved (Fig. 2A–C).

3.2.2. The effect of Forsythiae Fructus on intracellular ROS accumulation in IEC-6 cells

In normal cells, the expression of ROS is typically low. However, the administration of cisplatin often leads to an excessive ROS production, which is the prominent contributor to cellular damage.[23] To assess the antioxidant potential of Forsythiae Fructus, we examined intracellular ROS levels. As shown in Fig. 2G, treatment of IEC-6 cells with cisplatin resulted in a substantial increase in ROS production, which was mitigated by the combination of FAE, phillyrin, and forsythin A with NAC. To further confirm whether FAE, phillyrin, and forsythoside A could suppress oxidative stress, we employed tBHP, an exogenous inducer of oxidative stress, to treat IEC-6 cells. Exogenous tBHP was introduced during cell culture to induce oxidative stress in IEC-6 cells. In the case of tBHP-treated IEC-6 cells, a significant release of intracellular ROS was observed, which was effectively inhibited when FAE, phillyrin, and forsythoside A were combined with NAC (Fig. 2I).

3.2.3. The effect of Forsythiae Fructus on the morphology of cisplatin- or tBHP-treated IEC-6

Studies have shown that cisplatin is absorbed through passive

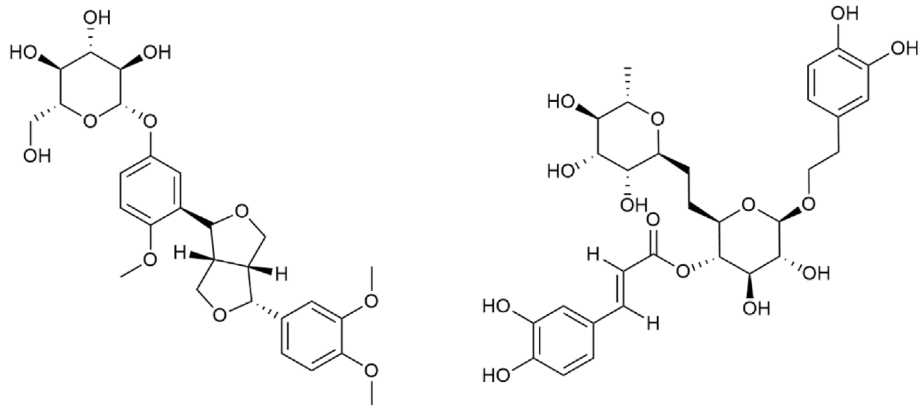

Fig. 1. The chemical structures of phillyrin ($C_{27}H_{34}O_{11}$) and forsythoside A ($C_{29}H_{36}O_{15}$).

diffusion and accumulates in the cytoplasm, mitochondria, and nucleus.[24] Cisplatin disrupts cell membranes, altering their fluidity and ultimately leading to cell death.[25] As part of our investigation into the cytotoxic effects of chemotherapeutic drugs on IEC-6 cells, we examined cell necrosis using Hoechst 33342/PI staining. In cisplatin-treated cells, we observed a strong red fluorescence (Fig. 2H), indicating cell necrosis. However, when FAE, phillyrin, and forsythoside A were combined with NAC, they significantly inhibited cell death caused by cisplatin. We conducted similar experiments with tBHP stimulation in IEC-6 cells. The results revealed increased necrotic cells following tBHP treatment (Fig. 2J). However, pretreatment of cells with NAC in combination with FAE, phillyrin, and forsythoside A resulted in a significant reduction in necrotic cells.

3.2.4. Forsythiae Fructus inhibits IEC-6 cell pyroptosis through the NLRP3/caspase-1/GSDMD-mediated signaling pathway

The overexpression of ROS activates NF-κB, which, in turn, promotes the maturation of cytokines pro-IL-1β and pro-IL-18, ultimately leading to the assembly of the NLRP3 inflammasome.[26] Next, we evaluated the effect of cisplatin or tBHP on IEC-6 cell pyroptosis. The expression of ASC, caspase-1, NLRP3, GSDMD-N, and HMGB1 in IEC-6 cells treated with cisplatin or tBHP increased. However, whenFAE, phillyrin, and forsythoside A were combined with NAC, there was a significant reduction in the expression of these proteins in IEC-6 cells stimulated with cisplatin or tBHP (Fig. 3A and B). The results were further confirmed using RT-PCR (Fig. 4). In comparison to the control group, the mRNA expression levels of *Asc*, *Nlrp3*, *Caspase1*, *Gsdmd*, *Hmgb1*, and *Il18* were abnormally elevated in the cells of the model group. However, the administration of FAE, phillyrin, and forsythoside A significantly reduced the mRNA expression levels of *Asc*, *Nlrp3*, *Caspase-1*, *Gsdmd*, *Hmgb1*, and *Il-18*. The results indicate that FAE alleviated NLRP3 inflammasome activation and pyroptosis induced by exposure to cisplatin or tBHP exposure.

In summary, these observations suggest that the combination of FAE, phillyrin, and forsythoside A with NAC can alleviate cisplatin or tBHP-induced cytotoxicity by inhibiting oxidative stress and reducing the NLRP3/caspase-1/GSDMD pathway in IEC-6 cells.

3.3. The effect of FAE on cisplatin or LPS/ATP-induced cytotoxicity in J774A.1 macrophages

3.3.1. The effect of Forsythiae Fructus on cell viability of cisplatin or LPS/ATP stimulated J774A.1 cells

To further understand the effect of inflammation on normal cells during chemotherapy, we performed similar studies as described above in J774A.1 macrophages. The IC50 of cisplatin or LPS/ATP was identified through a CCK-8 cell viability assay. J774A.1 macrophages exhibited approximately 50% cell viability after stimulation with 10 μM cisplatin or 1 μg/mL LPS/5 mM ATP. To establish the maximum threshold concentration of NAC, FAE, phillyrin, and forsythoside A, we evaluated their impact on macrophage J774A.1 viability. As shown in Supplementary Fig. 2, appropriate concentrations for NAC (0.01, 0.1, 1, and 10 μM), FAE (1×10^{-12}, 1×10^{-11}, and 1×10^{-10} g/L), phillyrin (0.001, 0.01, and 0.1 μM) and forsythoside A (0.1, 1, and 2.5 μM) were determined and used in subsequent experiments (Supplementary Fig. 2).

Next, we examined whether FAE, phillyrin, and forsythoside A could increase cell viability when J774A.1 macrophages were exposed to cisplatin or LPS/ATP. When FAE, phillyrin, and forsythoside A were administered alone to J774A.1 macrophage cells 1 h before cisplatin treatment, cell viability remained unchanged. However, cell viability significantly improved when FAE, phillyrin, and forsythoside A were combined with 10 μM NAC (Fig. 5A–F).

3.3.2. The effect of Forsythiae Fructus on intracellular ROS accumulation in J774A.1 macrophages

As shown in Fig. 5G and I, intracellular ROS levels significantly increased when cisplatin or LPS stimulated J774A.1 macrophages. However, the accumulation of ROS was reduced when FAE, phillyrin, and forsythoside A were combined with NAC, respectively. These results suggest that the concomitant use of Forsythiae Fructus with NAC has a mitigating effect on ROS overproduction in J774A.1 macrophages induced by cisplatin or LPS-ATP.

3.3.3. The effect of Forsythiae Fructus on the morphology of cisplatin- or LPS/ATP-treated IEC-6

As shown in Fig. 5H and J, compared to the normal control group, treatment with cisplatin or LPS/ATP significantly increased J774A.1 macrophage cell membrane rupture and compromised cell membrane integrity. This led to extensive necrosis in the macrophages. However, when compared to the model group, FAE, phillyrin, and forsythoside A combined with NAC resulted in a reduction in the number of necrotic cells.

3.3.4. Forsythiae Fructus inhibits J774A.1 cell pyroptosis through the NLRP3/caspase-1/GSDMD-mediated signaling pathway

We further explored the mechanism underlying the protective effects of FAE, phillyrin, and forsythoside A in combination with NAC on macrophages exposed to cisplatin or LPS/ATP. Western blotting analysis revealed that the NLRP3 inflammasome was activated, and the cell membrane was disrupted, leading to an increase in the expression of p–NF–κB, GSDMD-NT, caspase-1, IL-1β, and HMGB1 Fig. 6). These findings indicate that cisplatin or LPS/ATP can induce pyroptosis in

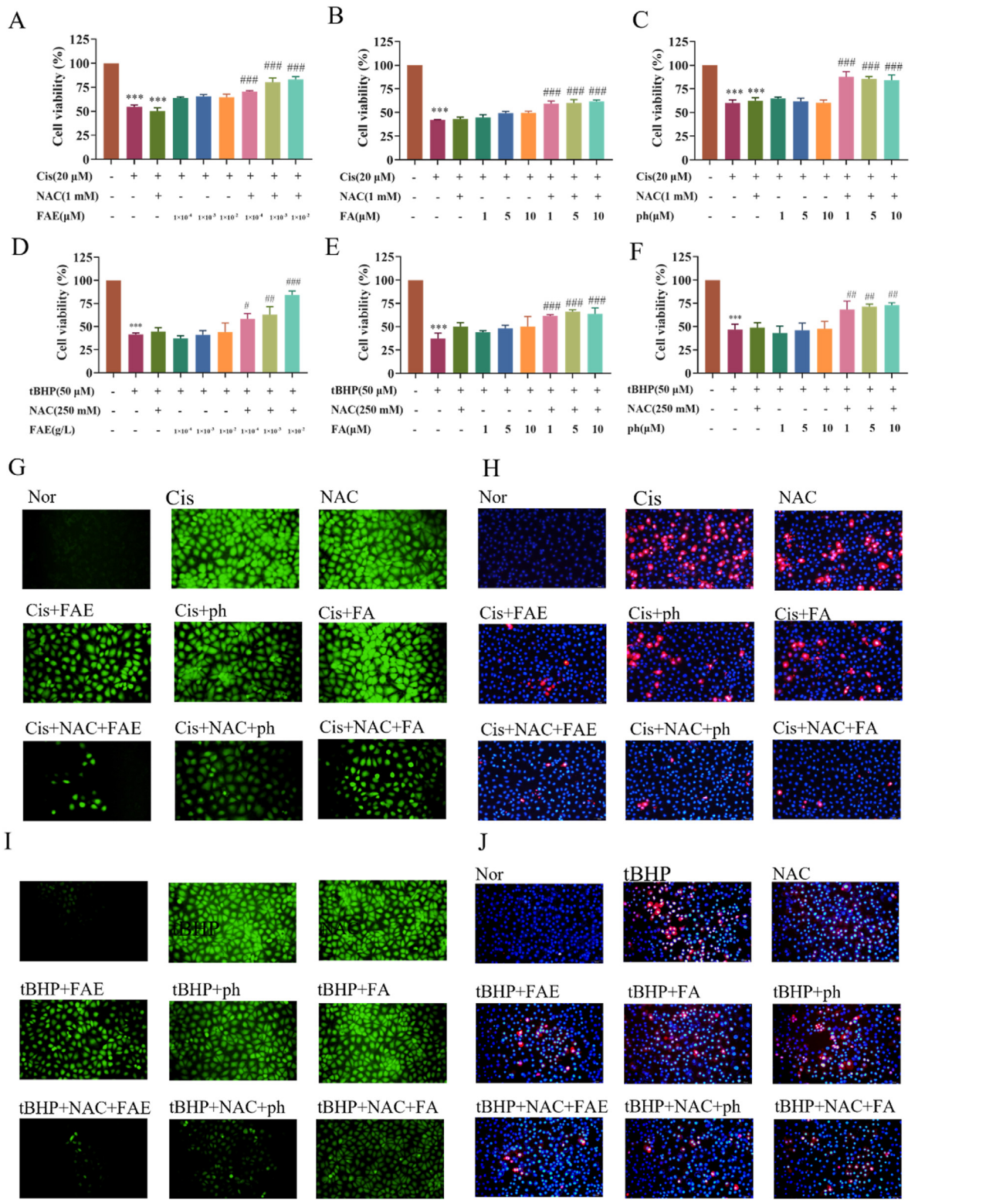

Fig. 2. Effect of Forsythiae Fructus on cisplatin or tBHP-induced IEC-6 cell injury.
(A–C) The effect of Forsythiae Fructus, forsythoside A, and phillyrin on the cell viability of cisplatin-induced IEC-6 cells. **(D–F)** The effect of Forsythiae Fructus, forsythoside A, and phillyrin on the cell viability of tBHP-induced IEC-6 cells. **(G and I)** Cisplatin or tBHP was added to cultured IEC-6 cells for 24 h after pretreatment with the test drugs for 1 h. The cells were then stained with 10 μM DCFH-DA for 20 min and observed under a fluorescent microscope. The green staining represents ROS levels. **(H and J)** After pretreatment with the test drugs for 1 h, cisplatin or tBHP was added to cultured IEC-6 cells for 24 h. The cells were stained with 5 μg/mL PI (red, staining dying cells) plus 5 μg/mL Hoechst 33342 (blue, staining all cells) for 25 min and observed by fluorescent microscopy. Photographs were taken under a fluorescence microscope at 200 × magnification (scale bar = 50 μm). All data are presented as the mean ± SEM (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared to the control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ compared to the cisplatin group. Cis, cisplatin; FAE, Forsythiae Fructus aqueous extract; ph, phillyrin; FA, forsythoside A; NAC, N-acetyl-L-cysteine. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

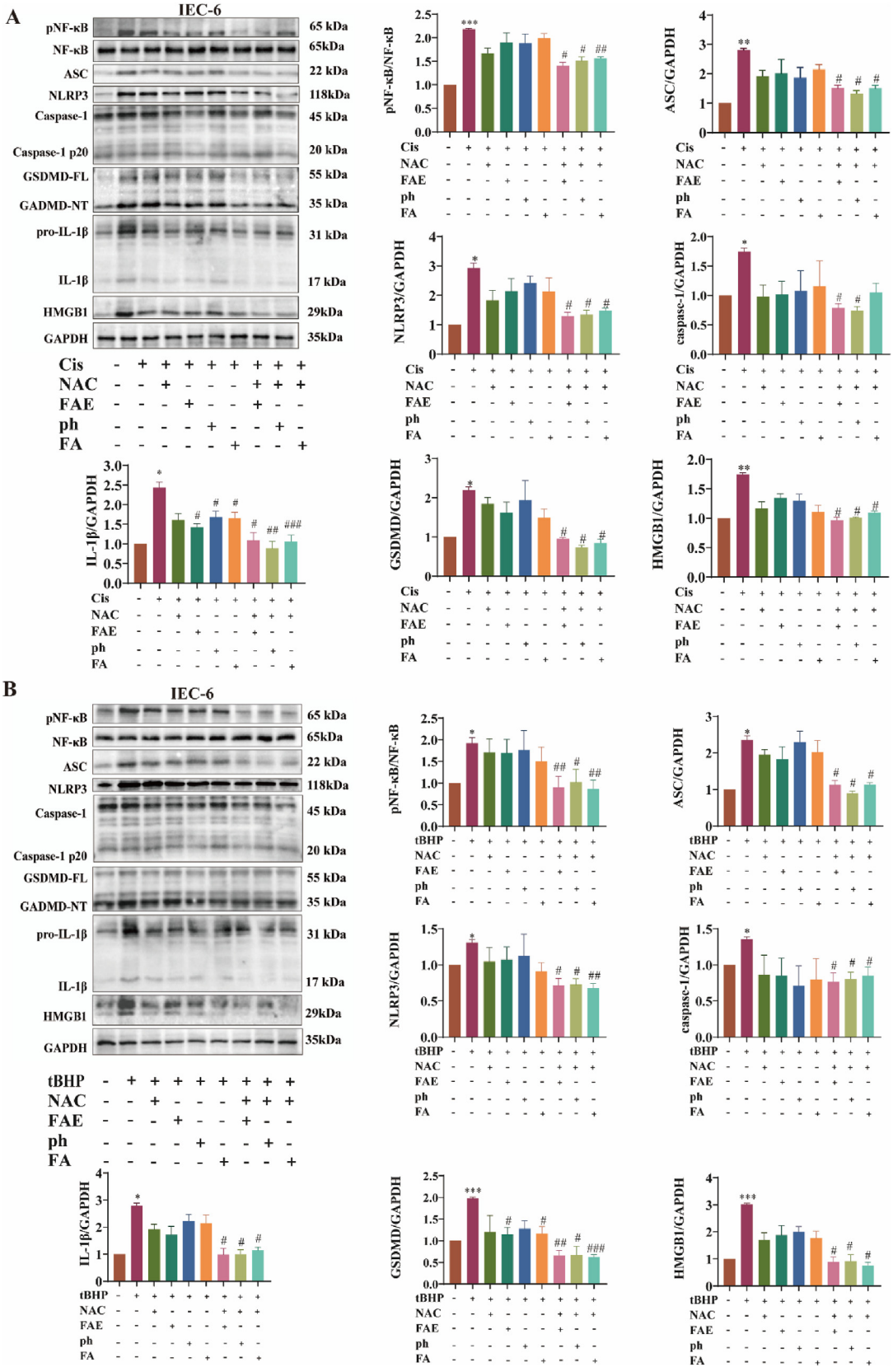

Fig. 3. Forsythiae Fructus inhibits cisplatin or tBHP-induced pyroptosis-related protein expression in IEC-6 cells.
Cisplatin (A) or tBHP (B) was added to cultured IEC-6 cells for 24 h following 1 h of pretreatment with the test drugs for 1 h. The expression levels of NLRP3, NF-κB, *p*-NF-κB, GSDMD-FL, DSDMD-NL, caspase-1, caspase-1p20, pro-IL-1*β*, IL-1*β*, HMGB1, and ASC were analyzed using western blotting. The ratios of NLRP3 to GAPDH, *p*-NF-κB to NF-κB, GSDMD-NT to GSDMD, caspase-1p20 to caspase-1, pro-IL-1*β* to IL-1*β*, HMGB1 to GAPDH, ASC to GAPDH were determined by the grayscale analysis of blots. All data are presented as the mean ± SEM (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, compared to the control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$, compared to the cisplatin group. Cis, cisplatin; FAE, Forsythiae Fructus aqueous extract; ph, phillyrin; FA, forsythoside A; NAC, N-acetyl-L-cysteine.

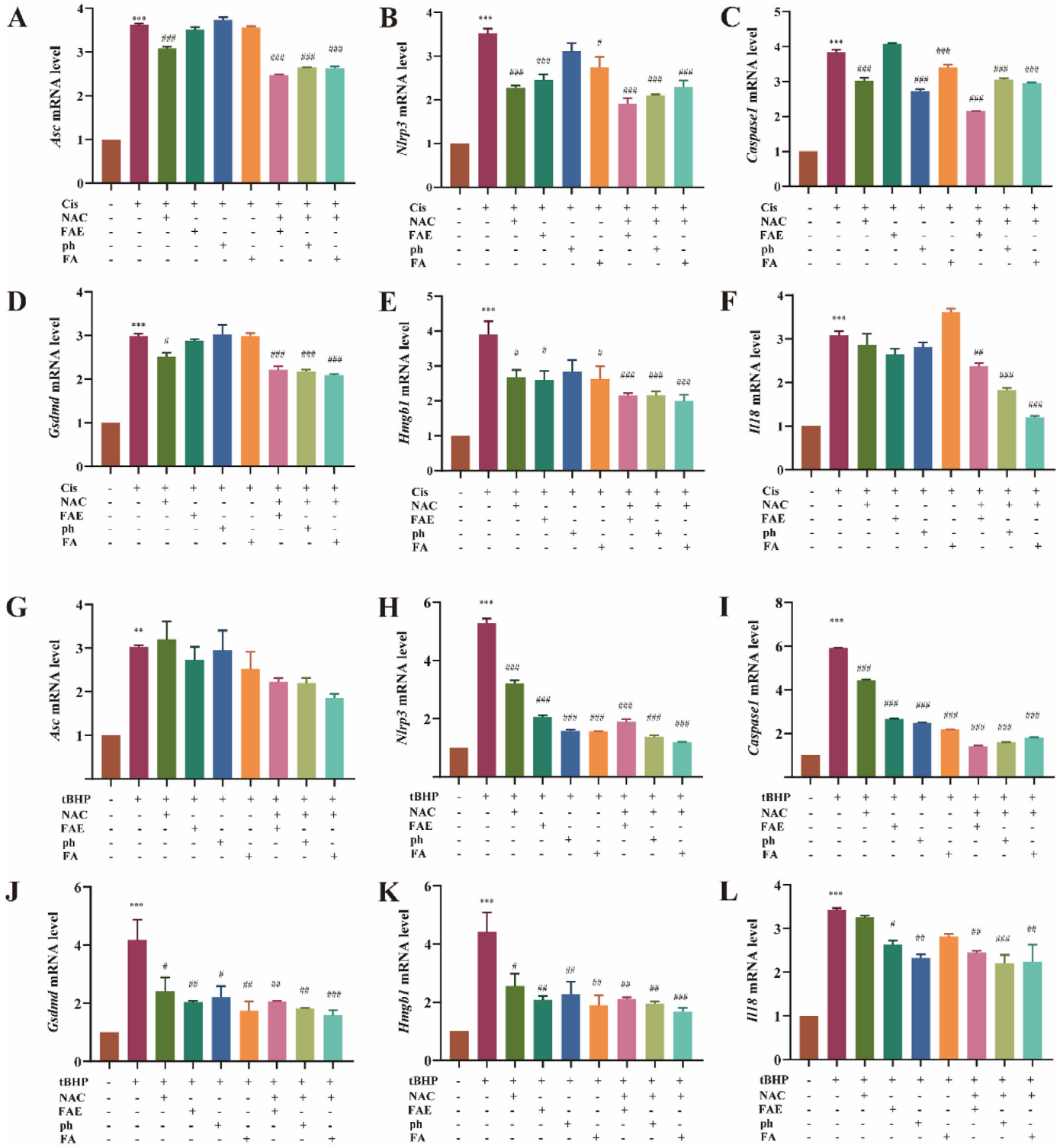

Fig. 4. Forsythiae Fructus inhibit cisplatin or tBHP-induced pyroptosis-related mRNA expression in IEC-6 cells.
(A–F) Effects of Forsythiae Fructus on the expression of mRNA related to cisplatin-induced pyroptosis in IEC-6 cells, (G–L) Effects of Forsythiae Fructus on the expression of mRNA related to tBHP-induced pyroptosis in IEC-6 cells. All data are presented as the mean ± SEM (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, compared to the control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$, compared to the cisplatin group. Cis, cisplatin; FAE, Forsythiae Fructus aqueous extract; ph, phillyrin; FA, forsythoside A; NAC, N-acetyl-L-cysteine.

J774A.1 macrophages. However, the combined treatment of FAE, phillyrin, and forsythoside A with NAC mitigated pyroptosis and inflammation. To enhance the reliability of the results, we also measured the mRNA levels of the related proteins, as shown in Fig. 7. In comparison to the control group, the expression levels of *Asc*, *Nlrp3*, *Caspase-1*, *Gsdmd*, *Hmgb1*, *Il-18*, and *Il1b* mRNA were abnormally elevated in the cisplatin or LPS/ATP-treated cells. However, when FAE, phillyrin, and forsythoside A were combined with NAC, there was a significant reduction in the expression of NLRP3, ASC, Caspase1, IL-18, GSDMD, and HMGB1.

In summary, FAE, phillyrin, and forsythoside A with NAC can

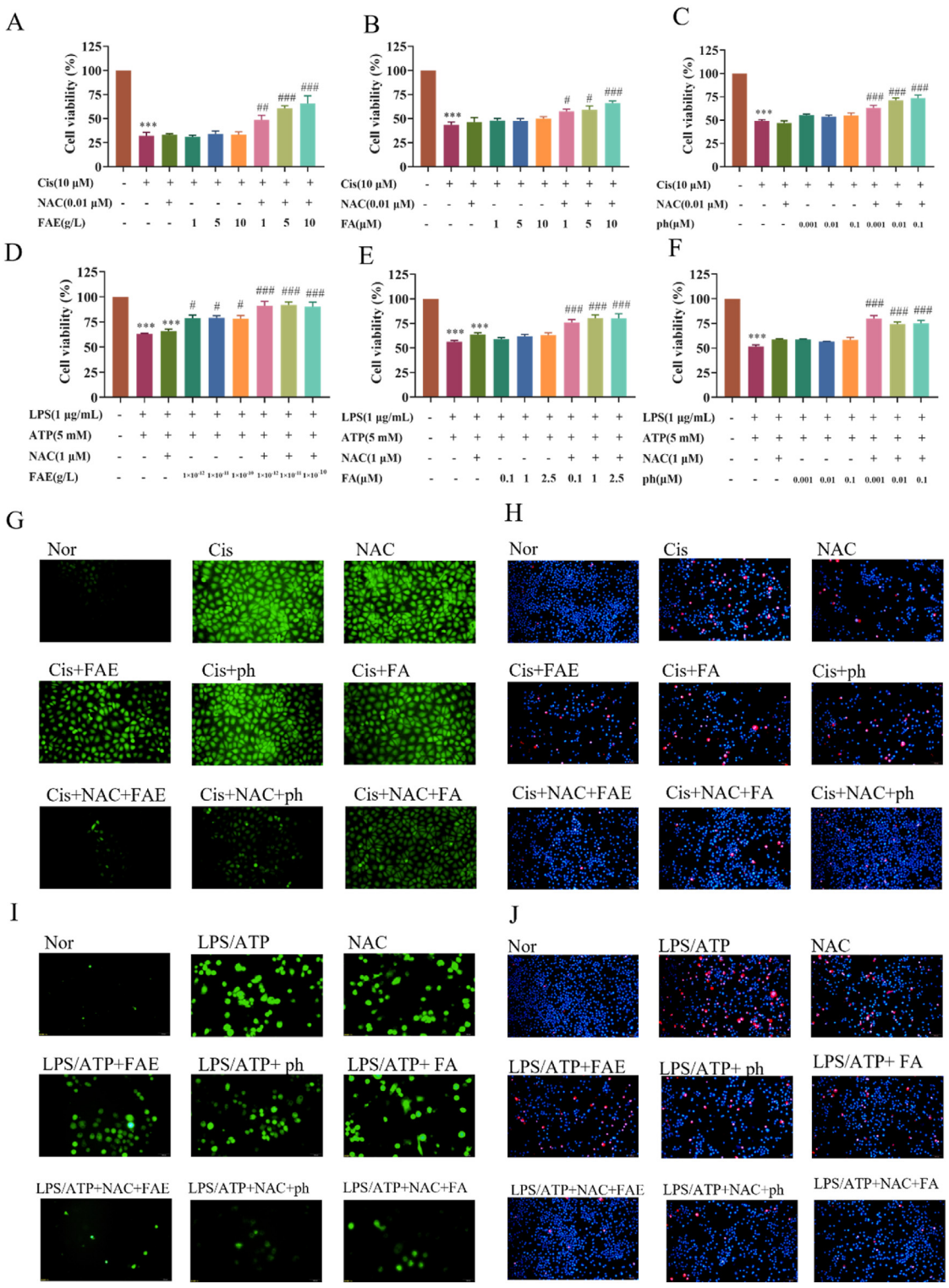

Fig. 5. Effect of Forsythiae Fructus on cisplatin or LPS/ATP-induced J774A.1 cell injury.
(A–C) The effects of Forsythiae Fructus, forsythoside A, and phillyrin on the cell viability of cisplatin-induced J774A.1 cells. **(D–F)** The effects of Forsythiae Fructus, forsythoside A, and phillyrin on the cell viability of LPS/ATP-induced J774A.1 cells. **(G and I)** Cisplatin or LPS/ATP was added to cultured IEC-6 cells for 24 h after pretreatment with the test drugs for 1 h. The cells were then stained with 10 μM DCFH-DA for 20 min and observed under a fluorescent microscope. The green staining represents ROS. **(H and J)** Cisplatin or LPS/ATP was added to cultured IEC-6 cells for 24 h after they were treated with the test drugs for 1 h. The cells were stained with 5 μg/mL PI (red, staining dying cells) and 5 μg/mL Hoechst 33342 (blue, staining all cells) for 25 min and observed under a fluorescent microscope. Photographs were taken under a fluorescence microscope at 200 × magnification (scale bar = 50 μm). All data are presented as the mean ± SEM (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, compared with the control group. $\#P < 0.05$, $\#\#P < 0.01$, and $\#\#\#P < 0.001$, compared to the cisplatin group. Cis, cisplatin; FAE, Forsythiae Fructus aqueous extract; ph, phillyrin; FA, forsythoside A; NAC, N-acetyl-L-cysteine. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

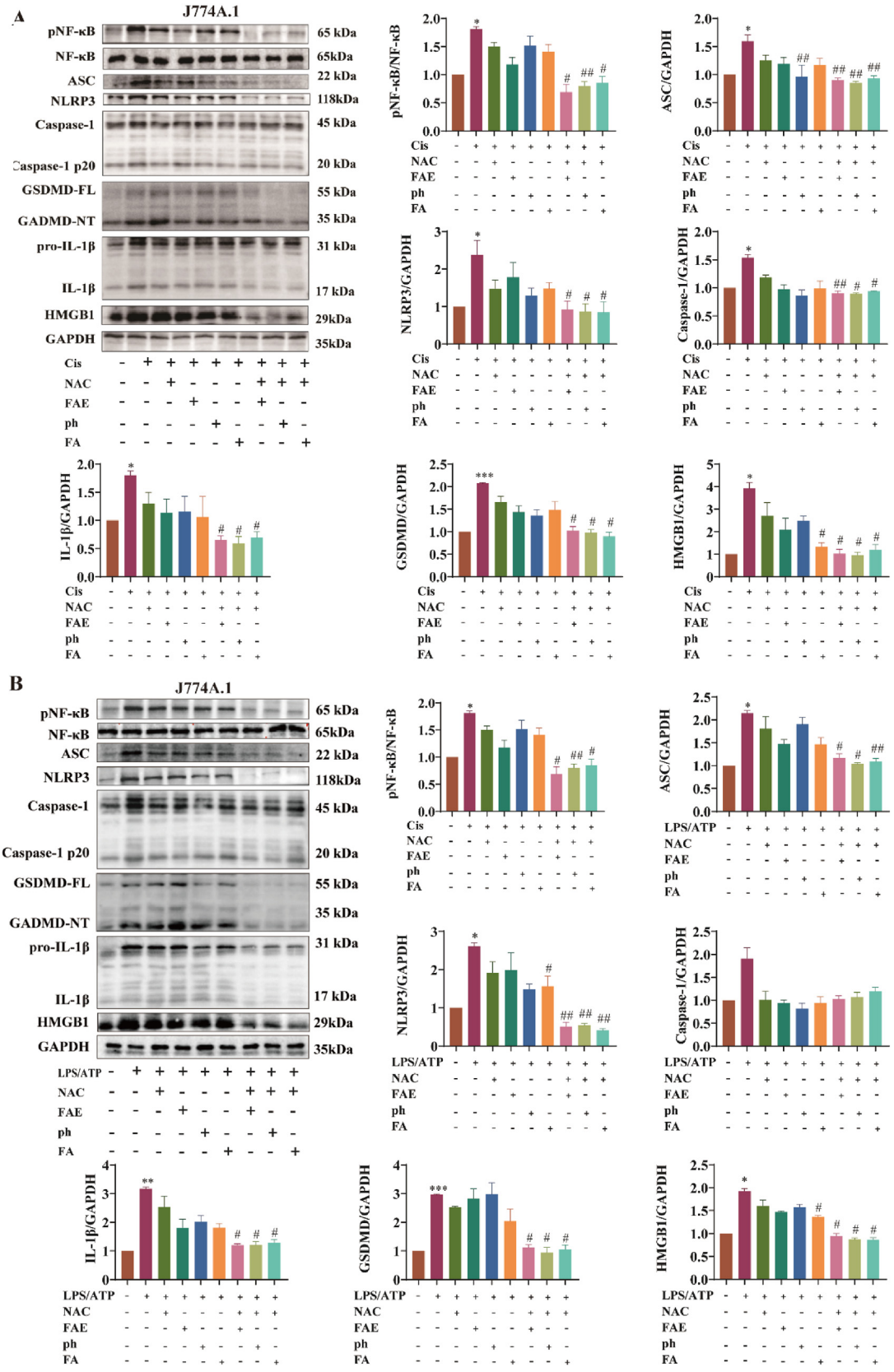

Fig. 6. Forsythiae Fructus inhibits cisplatin or LPS/ATP-induced pyroptosis-related protein expression in J774A.1 macrophages.
Cisplatin (A) or LPS/ATP(B) was added to cultured J774A.1 macrophages for 24 h after they were treated with the test drugs for 1 h. The expression levels of NLRP3, NF-κB, *p*-NF-κB, GSDMD-FL, DSDMD-NL, caspase-1, caspase-1p20, pro-IL-1*β*, IL-1*β*, HMGB1, and ASC were analyzed by western blotting. The ratios of NLRP3 to GAPDH, *p*-NF-κB to NF-κB, GSDMD-NT to GSDMD, caspase-1p20 to caspase-1, pro-IL-1*β* to IL-1*β*, HMGB1 to GAPDH, and ASC to GAPDH were analyzed by grayscale of blots. All data are presented as the mean ± SEM (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, compared to the control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$, compared to the cisplatin group. Cis, cisplatin; FAE, Forsythiae Fructus aqueous extract; ph, phillyrin; FA, forsythoside A; NAC, N-acetyl-L-cysteine.

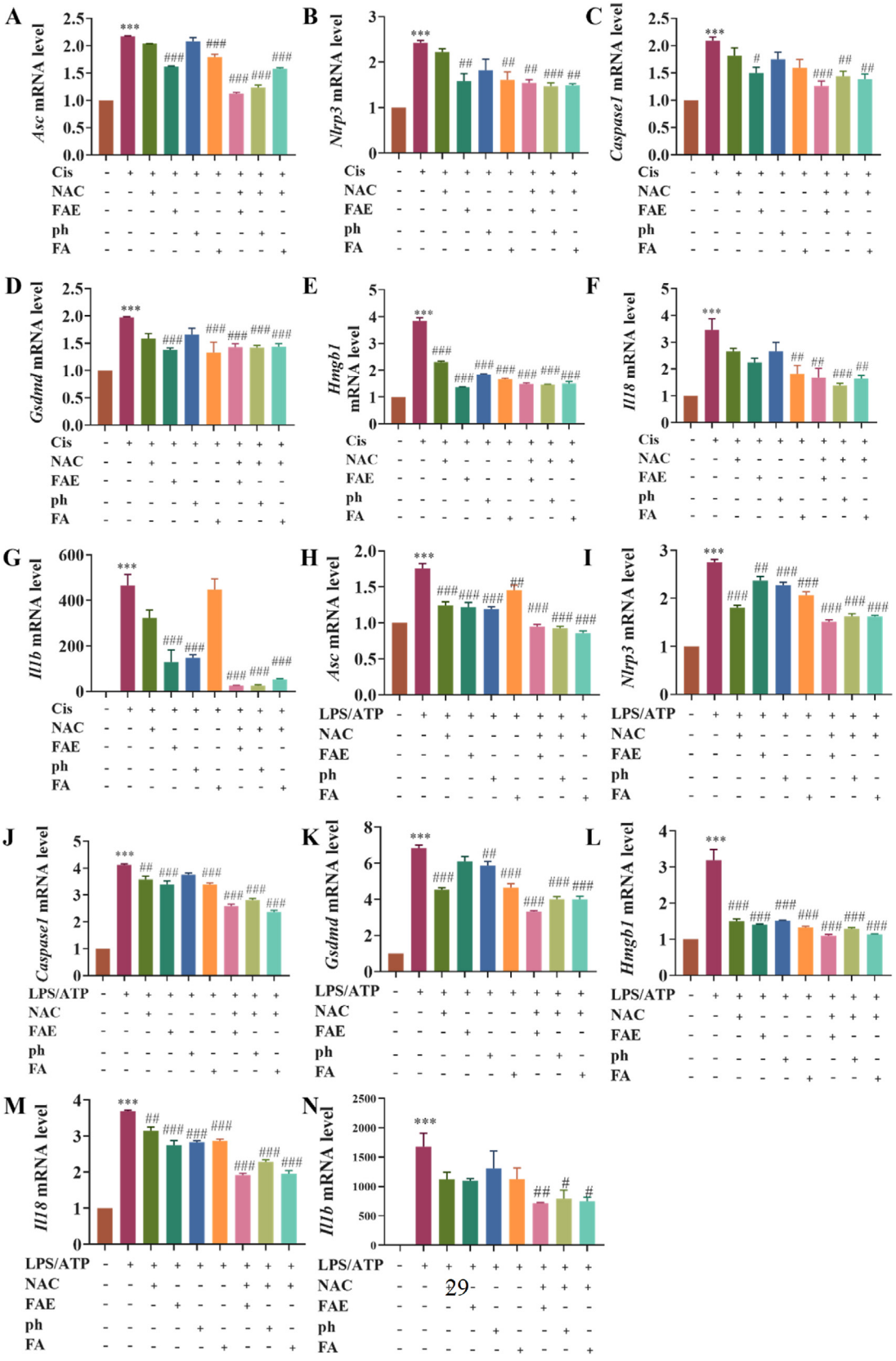

Fig. 7. Forsythiae Fructus inhibits cisplatin or LPS/ATP-induced pyroptosis-related mRNA expression in J774A.1 macrophages.
(A–G) Effects of Forsythiae Fructus on the expression of mRNA related to cisplatin-induced J774A.1 macrophages pyroptosis, (H–N) Effect of Forsythiae Fructus on the expression of mRNA related to LPS/ATP-induced J774A.1 macrophages pyroptosis. All data are presented as the mean ± SEM (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, compared to the control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$, compared to the cisplatin group. Cis, cisplatin; FAE, Forsythiae Fructus aqueous extract; ph, phillyrin; FA, forsythoside A; NAC, N-acetyl-L-cysteine.

alleviate cytotoxicity induced by cisplatin or LPS/ATP costimulation by inhibiting oxidative stress, reducing the NLRP3/caspase-1/GSDMD pathway-mediated pyroptosis in J774A.1 macrophages.

4. Discussion

CINV remains a prevalent adverse effect in patients undergoing antineoplastic therapy, significantly impacting their quality of life and often leading to reduced chemotherapy. Despite the availability of antiemetic medications, their efficacy is not absolute.[27] Therefore, there is a pressing need to enhance and optimize treatment strategies that address the pathogenesis of CINV from various angles. In earlier clinical trials, the steroidal anti-inflammatory drug dexamethasone demonstrated efficacy in treating CINV.[28] Additionally, preclinical trials indicated that dexamethasone could mitigate cisplatin-induced emesis in ferrets and cisplatin-induced pica in rats.[10,29] Furthermore, nonsteroidal anti-inflammatory drugs like indomethacin and meloxicam exhibited antiemetic properties against cisplatin-induced emesis in piglets.[30] These studies underscore the potential effectiveness of dampening chemotherapy-induced gastrointestinal inflammation as a strategy to combat CINV.

With the discovery of gasdermin's role in pyroptosis, investigators have confirmed that chemotherapy drugs can induce pyroptosis, primarily through GSDMD cleavage by caspase-3.[31] Furthermore, chemotherapy-induced inflammation in the gastrointestinal tract has been linked to GSDMD-mediated pyroptosis.[10] Recent studies have revealed that alleviating oxidative stress, inflammation, and pyroptosis can mitigate cisplatin-induced emesis.[23,32] Forsythiae Fructus, a traditional Chinese medicine known for its antioxidant and anti-inflammation properties, has previously shown antiemetic effects in CINV. One of the underlying mechanisms may involve the inhibition of cisplatin-induced gastrointestinal inflammation.[10] To gain a deeper understanding of its antiemetic mechanism, this study delves into the protective effects of Forsythiae Fructus and its active ingredients, phillyrin and forsythoside A, against cisplatin-induced cytotoxicity in IEC-6 cells and J774A.1 macrophages *in vitro*.

Intestinal epithelial cells, known for their rapid proliferation, are the primary target for damage by chemotherapeutic agents. During the destruction of the intestinal epithelium, necrotic epithelial cells release various proinflammatory factors, exacerbating gastrointestinal inflammation.[33] Therefore, protecting intestinal epithelial cells from injury is crucial in the treatment of gastrointestinal inflammation caused by chemotherapy. This study aims to investigate the mechanism of chemotherapy-induced gastrointestinal inflammatory injury, focusing on epithelial cells pyroptosis using cisplatin-treated IEC-6 cells. Upon cisplatin stimulation, we observed an increase in ROS production and the activation and assembly of NLRP3 inflammasome. Once activated, the NLRP3 inflammasome recruits procaspase-1, leading to its activation, cleavage of GSDMD, and subsequent formation of membrane pores. HMGB1 is released through these pores. Our findings indicate a strong connection between the development of gastrointestinal inflammation and intestinal epithelial cell pyroptosis induced by cisplatin.

ROS is a key trigger of chemotherapy-induced gastrointestinal inflammation,[23] and GSDMD-mediated pyroptosis is thought to involve the overproduction of ROS.[10] Our findings demonstrates that FAE and its main ingredients significantly reduce ROS levels in tBHP-treated IEC-6 cells, thereby countering pyroptosis and the release of inflammatory factors. These results suggest that inhibiting the ROS-NLRP3-GSDMD pathway is a key anti-inflammatory mechanism of FAE.

Gastrointestinal inflammation plays a significant role in the pathogenesis of CINV,[34] with macrophage contributing to this process. We assessed the toxicity of cisplatin and LPS/ATP on J774A.1 macrophages and evaluated the protective effect of FAE. Our results align with previous findings in IEC-6 cells, demonstrating that FAE significantly suppresses the activation of the NLRP3 inflammasome and GSDMD in cisplatin- and LPS/ATP-treated J774A.1 macrophages. This leads to a marked reduction in IL-1β and IL-18 levels following FAE treatment. These findings suggest a potential application of FAE in the treatment of gastrointestinal inflammation associated with pyroptosis.

In summary, pyroptosis in IEC-6 cells and J774A.1 macrophages induced by cisplatin contributes to increased production and release of inflammatory cytokines IL-1β and IL-18. NLRP3 inflammasome activation leads to caspase-1 activation, processing IL-1β and IL-18 into their active forms. Moreover, NLRP3 recognizes pathogens-associated molecular patterns and damage-associated molecular patterns, triggering GSDMD N-terminal pore formation in the cell membrane, which facilitates the release of IL-1β, IL-18, and HMGB1 from pyroptotic cells.[35] Elevated levels of IL-1β and IL-18 in cisplatin-induced cell death have been well-documented in numerous studies.[36–38] Importantly, recent research has implicated IL-1β in the pathogenesis of chemotherapy-induced gastrointestinal inflammation.[39] Therefore, alleviating gastrointestinal inflammatory injury by reducing pyroptosis and inflammatory cytokine release may contribute to Forsythiae Fructus' preventive effect against CINV.

To conclude, this study supports the idea that alleviating chemotherapy-induced gastrointestinal inflammation is a promising therapeutic approach against CINV. Our previous research in rats confirmed the anti-inflammation property and antiemetic efficacy of Forsythiae Fructus against cisplatin-induced gastrointestinal inflammation and CINV.[10] Building upon our previous studies, these *in vitro* experiments further show that treatment with Forsythiae Fructus and its main ingredients phillyrin and forsythoside A can improve the viability of cisplatin-treated IEC-6 cells and J774A.1 macrophages. Notably, the aqueous extract of Forsythiae Fructus exhibits superior efficacy compared to forsythoside A and phillyrin, likely due to the multiple bioactive components of Forsythiae Fructus that exert synergistic or potentiating effects, leading to complex biological responses. When combined, these components may produce therapeutic synergy, resulting in a more comprehensive anti-inflammatory or antioxidant effect. Of interest, cell viability substantially improves when Forsythiae Fructus or its main ingredients are used in combination with a subthreshold dose of NAC. Therefore, considering the combination of Forsythiae Fructus and NAC as a novel treatment for CINV warrants exploration in clinical trials.

5. Conclusion

The present study suggests that part of the protective mechanisms of Forsythiae Fructus against cisplatin-induced cytotoxicity involves inhibiting oxidative stress, downregulating the NLRP3/caspase-1/GSDMD pathway, and suppressing pyroptosis. When combined with our previous *in vivo* research,[10] these findings indicate that the antiemetic effects of Forsythiae Fructus in treating CINV may be associated with its ability to inhibit ROS-mediated oxidative stress, NLRP3 inflammasome activation, and caspase-1/GSDMD-mediated pyroptosis.

Authors' contributions

Conceived and designed the experiments: Ke Nie. Performed the experiments: Ruifang Zhang, Binbin Ye, Yihong Xian and Xiuxiu Liao. Analyzed the data: Binbin Ye and Ruifang Zhang. Wrote the manuscript: Binbin Ye. Revise for intellectual content: Ke Nie, Ruifang Zhang, Yihong Xian, Xiuxiu Liao and Weijian Chen. Funding acquisition: Ke Nie. All the listed authors have read and approved the submitted manuscript

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Declaration of competing interest

The authors declare no potential conflicts of interest associated with this research. All listed authors have read and approved the submitted manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhip.2023.09.001.

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