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Isosteroid alkaloids from *Fritillaria cirrhosa* bulbus as inhibitors of cigarette smoke-induced oxidative stress

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ABSTRACT

Fritillaria cirrhosa bulbus is a Chinese folk herb famous for its antitussive, expectorant, anti-asthma and anti-inflammatory properties, and is widely used to treat respiratory diseases. However, the impacts of *F. cirrhosa* bulbus on oxidative stress are still unkown. In the present study, we investigated the potential effect and mechanism of six isosteroid alkaloids with different chemical structures from *F. cirrhosa* bulbus on protection against cigarette smoke-induced oxidative stress in RAW264.7 macrophages. The results showed that six isosteroid alkaloids reduced reactive oxygen species (ROS) production, elevated glutathione (GSH) level and promoted heme oxygenase (HO-1) expression, which is in association with induction of NF-E2-related factor 2 (Nrf2) nuclear translocation and up-regulation of Nrf2 expression. Among these alkaloids, verticinone, verticine, imperialine-3-β-D-glucoside, delavine and peimisine exhibited more potent effect against CSE-induced oxidative stress than that of imperialine. These findings for the first time demonstrated that *F. cirrhosa* bulbus may play a protective role in cellular oxidative stress by activating Nrf2-mediated antioxidant pathway. Furthermore, the differences in antioxidant effects of these alkaloids were compared, as well as the corresponding structure-activity relationships were preliminarily elucidated. This suggested that *F. cirrhosa* bulbus might be a promising therapeutic treatment for the prevent of oxidative stress-related diseases.

1. Introduction

Oxidative stress is implicated in the pathogenesis of various respiratory diseases, such as acute lung injury, lung cancer, bronchitis, asthma, emphysema, pulmonary fibrosis and chronic obstructive pulmonary diseases (COPD) [1,2]. The generation of reactive oxygen species (ROS) is the chief characteristic of oxidative stress. Aberrant formation or accumulation of ROS can trigger sever cell damage and inflammatory injury, which aggravates the progression of lung diseases along with oxidant-antioxidant imbalance [3–5]. Cigarette smoke (CS), a complex mixture of toxic chemicals containing high levels of oxidants such as carbon monoxide (CO), nitric oxides and nicotine, is one of the most important risk factors of respiratory diseases [6,7]. Increased evidence indicated that long-term exposure to CS caused the activation of epithelial cells and macrophages in the lung, resulting in the occurrence of oxidant-antioxidant imbalance [8].

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a nuclear transcription factor that regulates the intracellular redox homeostasis after oxidants exposure, such as cigarette smoke [9]. Under oxidative stress, Nrf2 dissociates from its cytosolic repressor Kelch-like ECH-associated protein 1 (Keap1), translocates into the nucleus, binds to the antioxidant response elements (ARE) and subsequently regulate the

Abbreviation: COPD, chronic obstructive pulmonary diseases; ROS, reactive oxygen species; CS, cigarette smoke; CSE, cigarette smoke extract; CO, carbon monoxide; Nrf2, nuclear factor erythroid 2-related factor 2; Keap1, kelch-like ECH-associated protein 1; HO-1, heme oxygenase 1; GST, glutathione S-transferase; GCL, glutamate cysteine ligase; GSH, glutathione; T-GSH, total glutathione; GSSG, oxidized glutathione; DMEM, high-glucose Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PMSF, phenylmethylsulfonyl fluoride; DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DCFH-DA, 2′,7′-dichlorofluorescensin diacetate; DCF, dichlorofluorescein

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downstream genes expression for antioxidant and phase II detoxification enzymes, including heme oxygenase 1 (HO-1), glutathione S-transferase (GST), glutamate cysteine ligase (GCL), and so on [10–12]. Glutathione (GSH), an antioxidant molecular, plays a key role in neutralizing oxidants and maintaining the cellular redox balance [13]. It can be regulated by antioxidative enzymes such as GST and GCL [14]. Researches have also reported that inadequate level of GSH is associated with ROS-induced oxidative damage in pulmonary disorders [8]. In addition, it has been shown that ROS can be scavenged by Nrf2-activated endogenous enzymes, which suggests that Nrf2 signaling pathway may be a potent therapeutic target to enhance the capability of antioxidant defense [15].

In the lungs, macrophages play an important role not only in the maintenance of inflammatory-anti-inflammatory balance but also in oxidant-antioxidant balance. Previous researches have been reported that alveolar macrophages, are capable of inducing oxidant-mediated injury to lung parenchymal cells [16]. And a reduced expression of HO-1, associated with an altered expression of Nrf2 in alveolar macrophages in CS is related to severe airway diseases such as idiopathic pulmonary fibrosis, emphysema and COPD [16,17]. Numerous literatures have investigated the mechanisms for anti-oxidant response in CS-exposed lung cells including macrophages, which provide more clues for the treatment of respiratory diseases [18–21].

Fritillaria cirrhosa bulbus, belonged to the family of Liliaceae, has been commonly used in traditional Chinese medicine over two thousand years because of its low toxicity, remarkable therapeutic effects and less side effects [22]. Numerous pharmacological studies have reported that it was utilized to relieve various respiratory disorders, such as cough, expectoration, asthma, pneumonia, bronchial inflammation and acute lung injury, due to its antitussive, expectorant, anti-asthma, and anti-inflammatory activities, as well as anti-tumor activity [23,24]. Biological activities of *F. cirrhosa* bulbus are mainly attributed to its isosteroid alkaloids such as imperialine, chuanbeinone, verticinone, verticine, peimisine, and so on [25,26].

Recently, it has been reported that inflammation and oxidative stress are mutually involved in the pathological process of pulmonary diseases [27–29]. Previous studies only provided supports that total alkaloids extract or monomer alkaloids of *F. cirrhosa* bulbus is helpful to the treatment of respiratory inflammation. However, the therapeutic effect of *F. cirrhosa* bulbus on oxidative stress in the airways has not been studied. Therefore, this study investigated the potential of six isosteroid alkaloids from *F. cirrhosa* bulbus against cigarette smoke extract (CSE)-induced oxidative stress in RAW264.7 macrophages. The underlying molecular mechanisms were explored through observing the activation of Nrf2 signaling pathway. In addition, the different antioxidant efficacy of six alkaloids against oxidative stress were compared and elucidated preliminarily based on the differences in chemical structures.

2. Materials and methods

2.1. Materials and reagents

Imperialine, verticinone, verticine and peimisine were purchased from Push Bio-Technology (Chengdu, Sichuan, China). Imperialine-3-β-D-glucoside and delavine were purchased from Chengdu Herbpurify Co., LTD (Chengdu, Sichuan, China). The purity of all these substances is above 98%. RAW 264.7 murine macrophage cells were purchased from American Type Culture Collection (Manassas, VA, USA). High-glucose Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin solution were obtained from Hyclone (Logan, UT, USA). Phenylmethylsulfonyl fluoride (PMSF) and phosphatase inhibitor were purchased from Solaribo Life Sciences (Beijing, China). Dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Reactive Oxygen Species Assay Kit, GSH and GSSG Assay Kit and BCA protein kit were purchased from Beyotime Biotechnology (Shanghai, China). Mouse ELISA kits for HO-1 was purchased from Feiya Biotechnology (Jiangsu, China). Antibodies for Nrf2, Keap1, GAPDH and HRP-conjugated goat anti-rabbit IgG secondary antibody were obtained from Affinity Biosciences (OH, USA). The antibody for HO-1, β-actin and the goat anti-rabbit IgG/Alexa Fluor 488 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemical reagents used were of analytical grade.

2.2. Cell culture

RAW264.7 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37 °C in a humidified incubator containing 5% CO_2. In all experiments, the confluence of cells needs to be at 70–80% before exposure to any treatments.

2.3. CSE preparation

Commercial Huanghelou filtered cigarettes were obtained, and CSE extract was prepared as previous method with small modifications [30]. Briefly, the smoke from one cigarette was bubbling into 10 ml of serum-free DMEM to generate 10% of CSE. The CSE solution was adjusted to pH 7.4 and then sterile filtered through a 0.22 μm Millipore filter. CSE was freshly prepared for each experiment before use and diluted with culture media as required. Control medium was prepared by bubbling air through 10 ml of culture media, adjusting pH to 7.4, and sterile filtered as described above.

2.4. Cell viability assay

RAW264.7 cells were seeded in 96-well plates at 1.0×10^4 cells/well and treated with a serial dilutions (50, 40, 30, 20, 10, 5 μM) of six alkaloids (**A**-imperialine, **B**-verticinone, **C**-verticine, **D**-imperialine-3-β-D-glucoside, ***E***-delavine and **F**-peimisine) for 24 h. MTT solution (5.0 mg/ml) was then added into each well and the cells were incubated for another 4 h. The culture media was poured out, and 150 μl of DMSO was added to lyse crystals in each well. The absorbance was read at 490 nm on microplate reader (Bio-Rad, CA, USA).

2.5. Measurement of intracellular ROS level

Cellular ROS level was determined using fluorescent probe 2′,7′-dichlorofluorescensin diacetate (DCFH-DA). RAW264.7 cells were cultured in 24-well plates with a density of 9.0×10^5 cells/well for several hours. Then the cells were stimulated with a series of concentrations of CSE (0.25%, 0.5%, 1%, 2%, 5%) or six components with or without final CSE stimulus concentration for 24 h. Following this, the cells were incubated with DCFH-DA (10 μM) at 37 °C for 50 min in the dark. After incubation, the cells were washed with PBS twice and collected, subsequently measured the fluorescence intensity by flow cytometer (BD FACSCelesta, BD Biosciences, CA, USA) at the excitation and emission wavelengths of 488 nm and 525 nm, respectively.

2.6. Immunoflourescence of Nrf2 nuclear translocation

RAW264.7 cells on climbing slides were treated with indicated concentrations of six alkaloids in the presence or absence of CSE for 24 h. After the removal of culture medium, the cells were washed twice with PBS and fixed with 4% paraformaldehyde at room temperature for 30 min, followed by permeabilization with 0.1% Triton X-100 for 30 min. The cells were blocked with 5% goat serum for 20 min. Next, the cells were incubated with primary antibody for Nrf2 (1:200) overnight at 4 °C, followed by secondary antibody conjugated with Alexa Flour 488 (1:200) for 1 h at room temperature in the dark. After washing with PBS, DAPI (5.0 μg/ml) was used to stained the cell nuclei

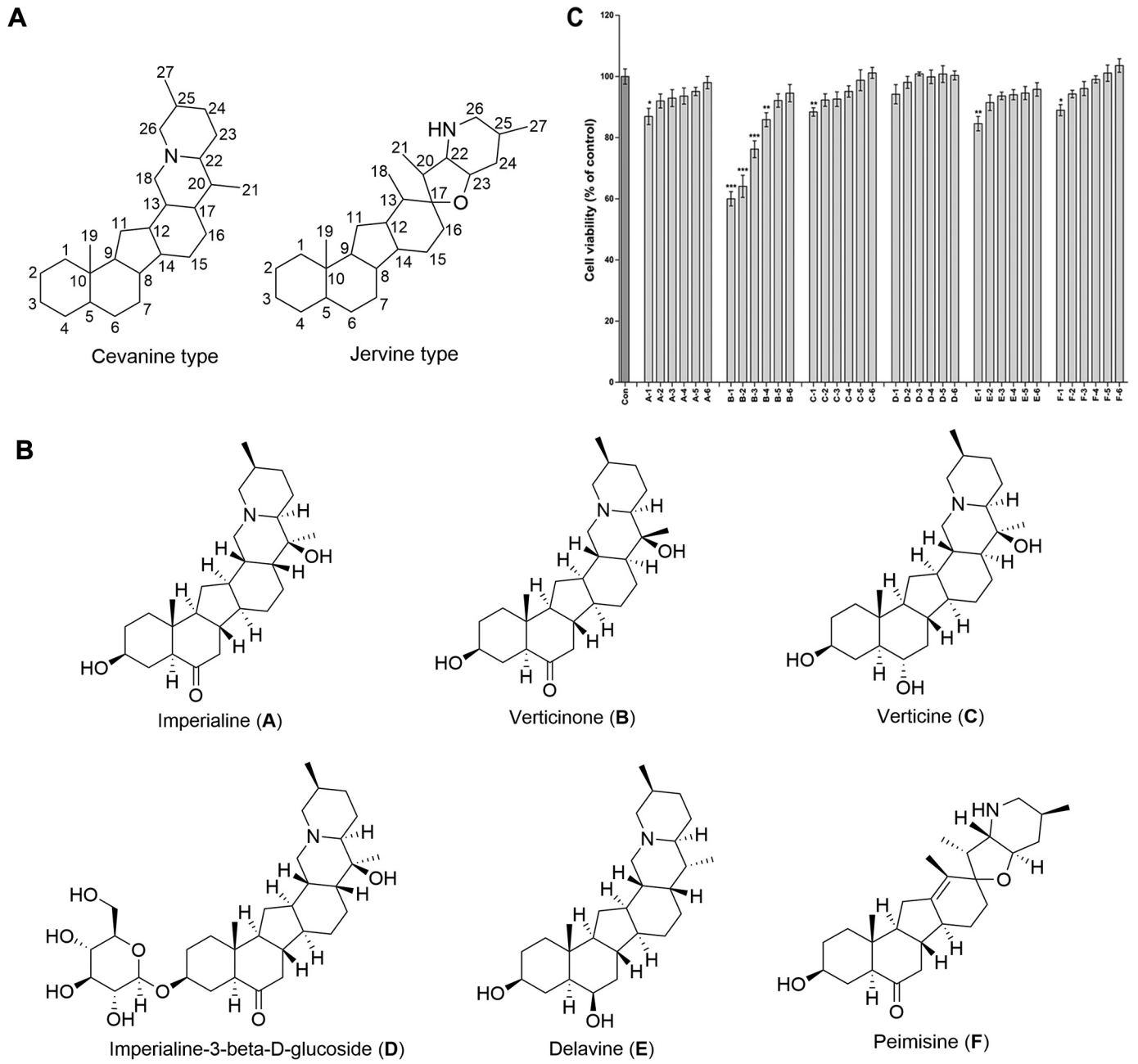

Fig. 1. Effects of six isosteroid alkaloids on the viability of RAW264.7 macrophages. (A) The alkaloid skeletons of cevanine type and jervine type. (B) The chemical structure of six alkaloids. (C) The cell viability data of six alkaloids. Cells were incubated with indicated concentrations of imperialine (**A**), verticinone (**B**), verticine (**C**), imperialine-3-β-D-glucoside (**D**), delavine (**E**) and peimisine (**F**) for 24 h. Numbers 1–6 represented the concentrations of 50, 40, 30, 20, 10 and 5 μM, respectively. Data were presented as the mean ± SEM of four independent experiments. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ *vs.* control.

for 5 min in the dark. Finally, the cells were observed with a laser scanning confocal microscope (Zeiss LSM510, Oberkochen, Germany).

2.7. Measurement of intracellular GSH levels

After treated with indicated concentrations of six alkaloids with or without CSE for 24 h, the amount of cellular total glutathione (T-GSH) and oxidized glutathione (GSSG) were measured by commercial GSH and GSSG assay kit, according to the manufacturer's instructions. Intracellular GSH levels were calculated as following: GSH = T-GSH − 2 × GSSG. The fluctuation of GSH level was precisely evaluated by GSH/GSSG ratio.

2.8. Enzyme-linked immunosorbent assay (ELISA) for HO-1

The level of HO-1 in the cell supernatant was determined by a quantitative ELISA kit according to the manufacturer's protocol.

2.9. Quantitative real-time PCR (qRT-PCR)

The total RNA was extracted (RNAprep pure Cell/Bacteria kit, Tiangen Biotech Co. LTD, Beijing, China) and quantified (NanoDrop 2000 spectrophotometer, Thermo Fisher Scientific, Waltham, USA). The purity was evaluated by A260/280 ratio. Total RNA (1 μg) was reverse transcribed to cDNA (PrimeScript™RT reagent Kit with gDNA Eraser, Takara, Osaka, Japan), which was then amplified using NovoStart SYBR qPCR SuperMix Plus (Novaprotein Scientific Inc.,

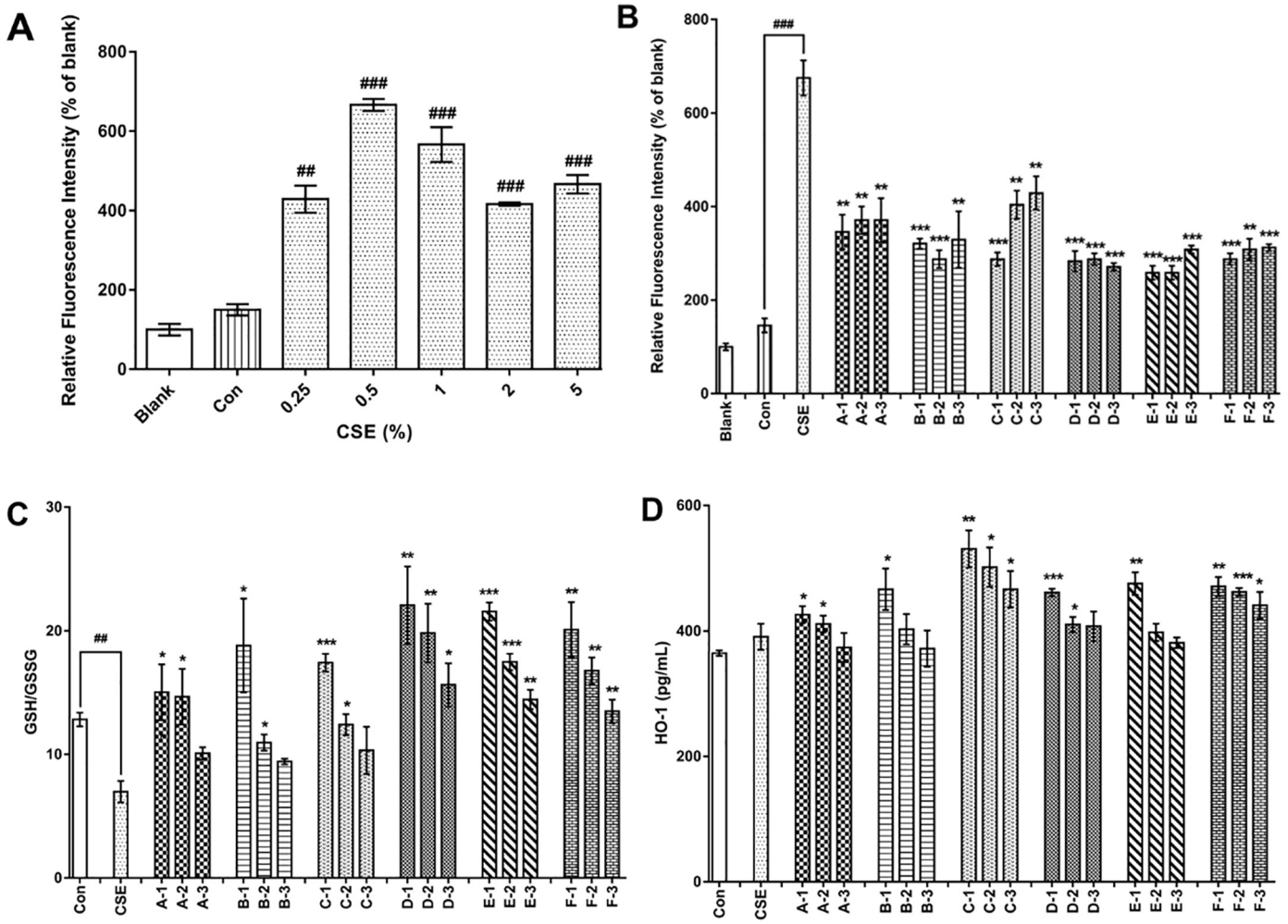

Fig. 2. Effects of six isosteroid alkaloids against CSE-induced oxidative stress in RAW264.7 macrophages. Cells were incubated with six alkaloids at indicated concentrations with or without CSE for 24 h. (A) The selection of CSE final stimulus concentration according to intracellular ROS production using DCFH-DA as a fluorescent probe. (B) Inhibition of six alkaloids on CSE-induced ROS production. (C) Increased effect of six alkaloids on GSH/GSSG ratio. (D) Elevated effect of six alkaloids on HO-1 level in cell supernatant. Data were presented as the mean ± SEM of three independent experiments. $^{\#\#}P < .01$, $^{\#\#\#}P < .001$ *vs.* control; $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ *vs.* CSE.

Jiangsu, China), performed with CFX Connect™ Real-Time PCR Detection System (BIO-RAD, US). The mRNA expression levels were calculated with the $2^{-\Delta\Delta Ct}$ method and normalized to the level of housekeeping gene GAPDH. The mouse GAPDH primers were purchased from Sangon Biotech (B661304, Shanghai, China). The other primers sequences used were as following: 5′-TCTGGATGGAGGGAGATACC-3′ (forward) and 5′-GGAAAGCCCATTTGAGTCCT-3′ (reverse) for HO-1; 5′- TCAGCGACAGAAGAACTAAG-3′ (forward) and 5′- AGGCATCTTGTTTGGGAATG-3′ (reverse) for Nrf2.

2.10. Western blotting analysis

RAW 264.7 cells were harvested and lysed using Nuclear and Cytoplasmic Protein Extraction Kit (Sangon Biotech, Shanghai, China) according to the manufacturer's instructions. The protein concentrations were determined by BCA protein assay kit. The nuclear proteins (60 μg) and cytoplasmic proteins (30 μg) were separated by 8% SDS-PAGE, respectively, and then electrotransferred to polyvinylidene difluoride filter (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% skim milk at room temperature for 1 h, followed by incubation overnight at 4 °C with primary antibodies for Nrf2 (1:1000), Keap-1 (1:2000), HO-1 (1:1000), GAPDH (1:20000) and β-actin (1:1000). Thereafter, the membranes were incubated with HRP-conjugated secondary antibody (1:5000) at 37 °C for 2 h. The protein bands were visualized using the Immobilon Western HPR Substrate (Millipore, Billerica, MA, USA) and images were captured using ChemiScope 6000 Touch Chemiluminescence Imaging System (CLiNX, Shanghai, China). The amount of protein was quantified using ImageJ software (National Institutes of Health, USA).

2.11. Statistical analysis

All data were presented as means ± standard error of mean (SEM). Statistical analysis was carried out using GraphPad Prism software 7.0 version (San Diego, CA, USA) with Student's *t*-tests. Significance was considered at $P < 0.05$.

3. Results

3.1. The chemical structure of six alkaloids

Six isosteroid alkaloids from *F. cirrhosa* bulbus, according to differences among their chemical structures, were divided into cevanine alkaloids and jervine alkaloids (Fig. 1A). As shown in Fig. 1B, the former includes imperialine (**A**), verticinone (**B**), verticine (**C**), imperialine-3-β-D-glucoside (**D**) and delavine (**E**), and the latter includes

peimisine (**F**).

3.2. Effect of six alkaloids on cell viability

The viability of RAW264.7 cells were determined by MTT assay. As shown in Fig. 1C, there was no significant effect of imperialine (**A**), verticine (**C**), delavine (**E**) and peimisine (**F**) on cell viability until the concentration reached 50 μM for 24 h. Noticeably, cell survival rate was not significantly affected by imperialine-3-β-D-glucoside (**D**) of 50 μM, but markedly decreased by verticinone (**B**) when the concentration only up to 20 μM. Based on these results, verticinone (**B**) of 10, 5, 2.5 μM was used in the subsequent experiments, as well as imperialine (**A**), verticine (**C**), imperialine-3-β-D-glucoside (**D**), delavine (**E**) and peimisine (**F**) of 40, 20, 10 μM.

3.3. Accumulation of ROS in RAW264.7 cells

To establish a model of CSE-induced RAW264.7 cells, we exposed the cells to 0.25%–5% CSE for 24 h and measured accumulation of ROS by DCFH-DA probe. The fluorescence intensity of dichlorofluorescein (DCF) corresponded to the intracellular ROS level. As shown in Fig. 2A, CSE was found to markedly increase ROS accumulation, and 0.5% CSE was selected as the final stimulus concentration to induce strongest oxidative stress in RAW264.7 cells. To investigated the effect of six isosteroid alkaloids against CSE-triggered ROS accumulation, the cells were co-incubated with indicated concentrations of six alkaloids with or without 0.5% CSE (Fig. 2B). After 24 h treatment, intracellular ROS levels were significantly decreased by six alkaloids compared to the CSE group. Among six alkaloids, verticine (**C**) of 10 μM and 20 μM showed relatively weaker effect on repression of ROS production, but not for 40 μM. Additionally, imperialine (**A**) did not inhibit ROS accumulation as strong as other compounds. The other alkaloids acted a similar inhibitory effect, which the reduction of the fluorescence intensity was closed to 50% in CSE-induced RAW264.7 cells.

3.4. Alterations of GSH/GSSG ratio and HO-1 levels

To evaluate whether six alkaloids alleviate CSE-induced oxidative stress, the GSH/GSSG ratio and antioxidant enzyme HO-1 level were determined. As shown in Fig. 2C, the GSH/GSSG ratio was lower in the CSE group than that in the control group. This suggested that 0.5% CSE significantly depleted GSH level in RAW264.7 cells. However, the depletion was reversed by six alkaloids with a significant increase in GSH/GSSG ratio in a dose-dependent manner, compared to the CSE group. At the concentration of 10 μM, verticinone (**B**) was observed the highest ratio of GSH/GSSG among six alkaloids. In addition, at the equivalent concentrations, imperialine-3-β-D-glucoside (**D**), delavine (**E**) and peimisine (**F**) displayed relatively stronger effect on the enhancement of GSH/GSSG ratio than that of other alkaloids. HO-1 is an inducible antioxidant enzyme which gets up-regulated in oxidative stress and provides potent anti-oxidant functions [31]. As shown in Fig. 2D, 0.5% CSE exposure induced slight up-regulation of HO-1 level but no statistic differences compared to the control group. Treatment with six alkaloids at the suitable concentrations significantly increased HO-1 level in a dose-dependent manner compared to the CSE group. Of which verticine (**C**) showed the highest elevation in HO-1 level, followed by peimisine (**F**) and 40 μM of delavine (**E**). At the concentration of 40 μM, imperialine (**A**) exhibited the mildest induction of HO-1 level among these alkaloids.

3.5. Nuclear translocation of Nrf2

Fluorescence assay was performed to determine the effect of six alkaloids on Nrf2 translocation. As shown in Fig. 3A, Nrf2 in the control group mainly concentrated in the cytoplasm and its expression was weak. Compared to the control group, stimulation by 0.5% CSE was slightly increased Nrf2 translocation from the cytoplasm to the nucleus, but not obvious. Treatment with six alkaloids significantly promoted the nuclear translocation of Nrf2 compared with CSE alone. Among six isosteroid alkaloids, when the concentration was at 40 μM, the facilitation of imperialine (**A**) on Nrf2 nuclear translocation was not so remarkable, but the other alkaloid, including verticinone (**B**) of 10 μM, showed a predominant enhancement in Nrf2 translocation from the cytoplasm to the nucleus.

3.6. Regulations of Nrf2 and HO-1 mRNA expression

RT-PCR assay was carried out to determine the effect of six alkaloids on the mRNA levels of Nrf2 and HO-1 in response to CSE-induced oxidative stress. Compared to the control group, the mRNA expressions of Nrf2 and HO-1 were significantly upregulated in the CSE group (Fig. 3B, Fig. 3C). Likewise, treatment with six alkaloids markedly increased Nrf2 and HO-1 mRNA expressions compared to the CSE group. Of which the highest levels of Nrf2 and HO-1 mRNA were both observed in 40 μM of verticine (**C**) treatment group. Also, imperialine-3-β-D-glucoside (**D**), delavine (**E**) and peimisine (**F**) displayed comparatively stronger effects to enhance the mRNA expression of Nrf2 and HO-1 at the concentration of 40 μM. However, the increased mRNA levels of Nrf2 and HO-1 were relatively lower in imperialine (**A**) treatment group, especially at 40 μM. These results were coincident with Nrf2 nuclear translocation and upregulation of HO-1 level, which suggested that six alkaloids may exert protective effect in CSE-induced oxidative stress by induction of Nrf2 signaling pathway and enhancement of its downstream gene expression.

3.7. Protein expression of Nrf2 signaling pathway

To further investigate whether six alkaloids modulate oxidative stress though Nrf2 signaling pathway, the immunoblotting analysis was performed. As shown in Fig. 3D, Fig. 3E, in the CSE group, no apparent increase of Nrf2 protein level was observed in the nucleus, while the nuclear level of Nrf2 protein was effectively elevated by six alkaloids at the right concentrations. In sum, this result was basically consistent with that of Nrf2 nuclear translocation with the comparatively weaker effect in 40 μM of imperialine (**A**). As shown in Fig. 3D, Fig. 3F, 0.5% CSE did not affect the expression of Keap1 protein compare with the control group. Similarly, there was no significant change on Keap1 expression in six alkaloids groups, except for delavine (**E**) and peimisine (**F**) at the concentration of 40 μM. To confirm activation of Nrf2 induced HO-1expression, we investigated the protein level of HO-1 in CSE-induced RAW264.7 cells. As shown in Fig. 3D, Fig. 3G, the expression of HO-1 protein did not markedly change after the cells were exposed to CSE for 24 h. Whereas, treatment with six alkaloids resulted in a significant upregulation of HO-1 protein level. Among six alkaloids, the HO-1 protein expression in imperialine (**A**) group was relatively lower than that of any groups. The other alkaloids all showed a relatively pronounced increase in HO-1 protein expression. These results were accordance with the upregulation of HO-1mRNA level.

4. Discussion

Isosteroid alkaloids, widely distributed in the bulbus of *F. cirrhosa*, have broad biological activities, which is responsible for the traditional use of *F. cirrhosa* bulbus in pulmonary diseases. In recent years, numerous literatures have reported that inflammation and oxidative stress are collectively involved in the pathogenesis of airway diseases, such as lung injury, pneumonia, pulmonary fibrosis, asthma and COPD [27–29,32]. In previous studies, isosteroid alkaloids from *F. cirrhosa* bulbus exhibited a satisfactory anti-inflammatory activity to suppress inflammatory gene expression and inflammatory cytokines release in cells and mice [33–35]. Nevertheless, there have been few reports about the beneficial effects of *F. cirrhosa* bulbus against oxidative

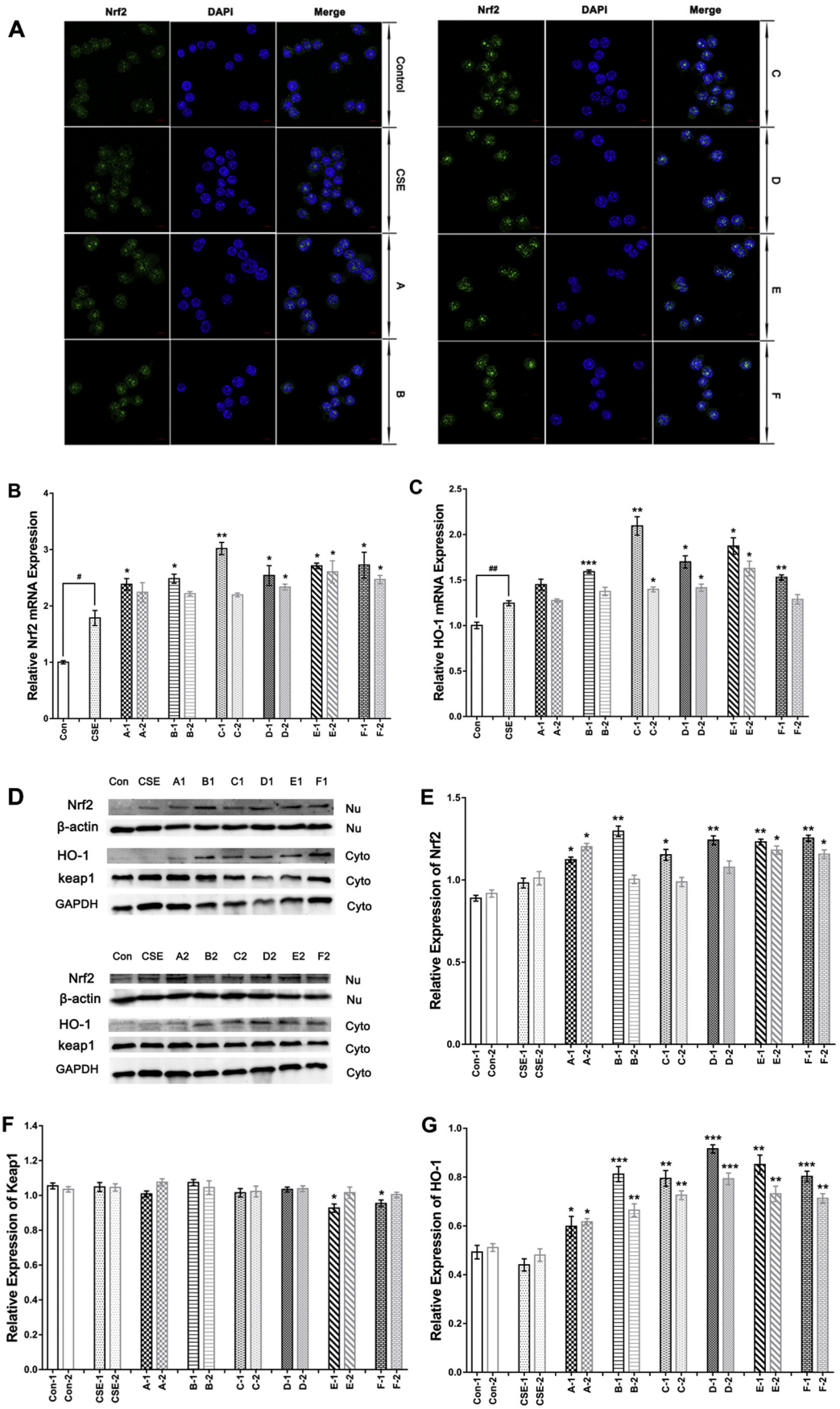

Fig. 3. Effects of six isosteroid alkaloids on activation of Nrf2 signaling pathway in CSE-induced RAW264.7 macrophages. Cells were incubated with six alkaloids at indicated concentrations with or without CSE for 24 h. (A) Nuclear translocation of Nrf2 was determined by immunofluorescence staining. The scale bar was 50 μM. Six alkaloids promoted Nrf2 translocation from the cytoplasm into the nucleus. (B and C) Upregulation of six alkaloids on the mRNA expression of Nrf2 and HO-1. (D, E, F and G) Effect of six alkaloids on Nrf2, Keap1 and HO-1 protein expressions. The protein expression of Nrf2, Keap1 and HO-1 was detected by western blotting analysis. The protein bands were quantified by integrated optical density (IOD) using Image J software and normalized to β-actin or GAPDH. Data were presented as the mean ± SEM of three independent experiments. $^{\#}P < .05$, $^{\#\#}P < .01$ *vs.* control; $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ *vs.* CSE.

insults. Cigarette smoke, as the most common oxidative irritants, lead to a large amount of intracellular ROS generation, following by the imbalance between oxidants and antioxidants. Exposure to CS, macrophages are one of important cells to be activated in response to oxidative insults in the airways. Currently, more and more researches have also studied the protective effects of natural products or drugs against CS or CSE-induced oxidative stress in macrophages not only in epithelial cells, so as to well illustrate the correlation between inflammation and oxidative stress in lung diseases [18,21,36–38]. Therefore, based on previous studies of anti-inflammatory effects, we investigated the protective effects of isosteroid alkaloids from *F. cirrhosa* bulbus on CSE-triggered oxidants-antioxidants imbalance in RAW264.7 macrophages.

In this study, we determined the therapeutic concentrations of six alkaloids at first. The results showed that the cytotoxicity of verticinone (**B**) was the highest, while imperialine-3-β-D-glucoside (**D**) displayed the weakest cytotoxicity among the six alkaloids. Compared to the differences of six alkaloids on chemical structures, we speculated that the presence of β-CH_3 at C-20 position may hugely elevate the cytotoxicity of isosteroid alkaloids. The lower cytotoxicity of imperialine-3-β-D-glucoside (**D**) may be caused by the substitution of glucoside at C-3 position. In addition, there was little difference in cytotoxicity between alkaloid skeletons of cevanine types and jervine types. Based on above observations, cytotoxicity of isosteroid alkaloids is more dependent on the functional groups rather than alkaloid skeleton types.

Increasing evidence indicated that ROS has wide-ranging effects in airway pathology, and reducing ROS production contributes to the alleviation of oxidative stress and subsequent the improvement of disease conditions [39]. In our results, exposure to CSE led to increased production of ROS in RAW264.7 macrophages, suggesting that CSE induced oxidative stress by generating excessive ROS. This was consistent with previous studies [40]. Six isosteroid alkaloids significantly attenuated CSE-stimulated ROS generation. The relatively weaker inhibition at the concentration of 10 μM and 20 μM, but dramatically stronger at 40 μM demonstrated that verticine (**C**) had a strong dose-dependent manner on suppression of ROS. Also, the ROS inhibitory effect of imperialine (**A**) was comparatively lower among other alkaloids. The oxidants-antioxidants imbalance is also associated with the reduction of GSH and the increase of GSSG. Recent studies suggested that the stimulation of GSH synthesis, the inhibition of GSH depletion and the suppression of GSSG generation are major contributors to the antioxidant response [41]. Our results showed that six isosteroid alkaloids significantly improved intracellular GSH/GSSG ratio, which means they can increase GSH level and/or decreased GSSG level. The higher GSH/GSSG ratio was observed in imperialine-3-β-D-glucoside (**D**), delavine (**E**) and peimisine (**F**) treated groups, which could be correlated with the presence of glucoside at C-3 position, the deletion of β-OH at C-20 position and the substitution of Funan ring at C-17 position, respectively. Under the same concentration of 10 μM, the strongest effect was found in verticinone (**B**) group. It suggested that the presence of β-CH_3 at C-20 position not only affected the cytotoxicity of verticinone (**B**), but also determined its favourable efficacy.

HO-1 is an antioxidant enzyme induced by oxidative stress, and gets overexpressed to protect cells from oxidative damage and help maintain redox homeostasis [42]. Due to its cytoprotective capability, induction of HO-1 has been considered as a potential therapeutic target against oxidative insults. In the present study, six alkaloids increased the HO-1 level by upregulating the mRNA and protein expression of HO-1 at the right concentrations. Also, the change of HO-1 level in cell supernatant was almost in correspondence with the mRNA and protein levels of HO-1. From the perspective of structure-activity relationship, we preliminarily deduced that the substitution of –OH at C-3 position played a central role in enhancing induction of HO-1, such as verticine (**C**) and delavine (**E**). However, the enhancement was heavily weakened by the substitution of = O instead of –OH at C-3 position, such as imperialine (**A**). Although verticinone (**B**) has the same founctional groups as imperialine (**A**), its effect on induction of HO-1 was not seriously slacked, which may be caused by the isomerism of functional groups, such as α-H at C-17 position and β-CH_3 at C-20 position. Interestingly, imperialine-3-β-D-glucoside (**D**) and peimisine (**F**) exerted relatively stronger effect on upregulation of HO-1 although the presence of = O at C-3 position, which implied that the weakening effect could be counteracted and reversed by the substitution of glucoside at C-1 position and unique structure characteristic of jervine alkaloid skeleton.

Nrf2 is an important transcription factor to regulate intracellular oxidative stress through activating target gene transcriptions of detoxification and antioxidant enzymes, including heme oxygenase 1 (HO-1), glutathione S-transferase (GST) and glutamate cysteine ligase (GCL), thereby reducing ROS production and increasing GSH level [43,44]. A considerable number of reports suggested that Nrf2 plays a key role in protection against CS or CSE-induced oxidative stress in the lungs [45–47]. A decline in Nrf2 level is closely correlated with the severity of lung diseases along with an increase in oxidative stress [48,49]. It has been also showed that knockdown of Nrf2 caused the aggravation of lung diseases by downregulating the expression of antioxidant genes [8,50]. Based on our above results, we concluded that the underlying molecular mechanism of six alkaloids against CSE-induced oxidative stress may contribute to the modulation of Nrf2 signaling pathway. The immunofluorescence results showed that six alkaloids induced translocation of Nrf2 into the nucleus, which was verified by western blot analysis. It was revealed that six alkaloids significantly upregulated the expression of nuclear Nrf2 protein in CSE-exposed RAW264.7 cells. Consistent with these results, the mRNA level of Nrf2 was also significantly increased by six alkaloids. These data demonstrated that six alkaloids can activate Nrf2 signaling pathway though promoting Nrf2 nuclear translocation, increasing Nrf2 mRNA expression and elevating Nrf2 protein level. Moreover, at the concentration of 40 μM, verticine (**C**), imperialine-3-β-D-glucoside (**D**), delavine (**E**) and peimisine (**F**) had comparatively stronger activation on Nrf2 than that of imperialine (**A**). Same strong effect was also found in 10 μM of verticinone (**B**). On the other hand, although earlier studies have been reported that the activation of Nrf2 was associated with the inhibition of Keap1, there was no significant downregulation of Keap1 protein in the current study, except for delavine (**E**) and peimisine (**F**) of 40 μM, which could be related to the lack of β-OH at C-20 position and chemical structure characteristic of jervine alkaloid skeleton, respectively.

5. Conclusion

In conclusion, this study for the first time demonstrated that six isosteroid alkaloids (imperialine, verticinone, verticine, imperialine-3-β-D-glucoside, delavine and peimisine) from *F. cirrhosa* bulbus effectively protected against CSE-induced oxidative stress in RAW264.7 macrophages by suppressing ROS accumulation, increasing GSH level and improving HO-1 expression.. The potential mechanism maybe related to activation of Nrf2 signaling pathway. Furthermore, the structure-activity relationship of six compounds was preliminarily elucidate. The effect of imperialine on anti-oxidative stress was relatively weaker than that of other alkaloids. Verticinone, verticine, imperialine-3-β-D-glucoside, delavine and peimisine exhibited more potent effect against CSE-induced oxidative stress. Thus, the present study provided fundamental evidence that *F. cirrhosa* bulbus could be a promising alternative for treatment of oxidative stress in lungs. However, the specific mechanism of Nrf2 activation needs to be explored in the further studies. And it also needs to investigate whether the combination action of multiple alkaloids is more effective than that of a single alkaloid in *F. cirrhosa* bulbus.

Author statement

All authors agreed to submit the enclosed manuscript entitled "**Isosteroid alkaloids from *Fritillaria cirrhosa* bulbus as inhibitors**

of cigarette smoke-induced oxidative stress" to "FITOTERAPIA", and revise it according to the comments of reviewers. No conflict of interest exits in the submission of this manuscript. We would like to declare that the work described was original research that has not been published previously, and not under consideration for publication elsewhere, in whole or in part. All the authors listed have approved the manuscript that is enclosed.

Declaration of Competing Interest

None.

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