Research Article

4,7-Didehydro-neophysalin B Protects Rat Lung Epithelial Cells against Hydrogen Peroxide-Induced Oxidative Damage through Nrf2-Mediated Signaling Pathway

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Received 4 June 2021; Revised 29 June 2022; Accepted 19 July 2022; Published 12 September 2022

Academic Editor: Anderson J. Teodoro

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The administration of 4,7-didehydro-neophysalin B is expected to be a promising strategy for mitigating oxidative stress in respiratory diseases. This study was aimed at investigating the efficacy of 4,7-didehydro-neophysalin B for apoptosis resistance of rat lung epithelial cells (RLE-6TN) to oxidative stress and evaluating its underlying mechanism of action. The RLE-6TN cells treated with hydrogen peroxide (H₂O₂) were divided into five groups, and 4,7-didehydro-neophysalin B was administered into it. To evaluate its mechanism of action, the expression of oxidative stress and apoptotic proteins was investigated. 4,7-Didehydro-neophysalin B significantly inhibited H₂O₂-induced RLE-6TN cell damage. It also activated the Nrf2 signaling pathway which was evident from the increased transcription of antioxidant responsive of KLF9, NQO1, Keap-1, and HO-1. Nrf2 was found to be a potential target of 4,7-didehydro-neophysalin B. The protein levels of Bcl-2 and Bcl-xL were increased while Bax and p53 were decreased significantly. Flow cytometry showed that 4,7-didehydro-neophysalin B protected RLE-6TN cells from apoptosis and has improved the oxidative damage. This study provided a promising evidence that 4,7-didehydro-neophysalin B can be a therapeutic option for oxidative stress in respiratory diseases.

1. Introduction

The respiratory epithelial cell damage is a key indicator of respiratory diseases. Investigation of respiratory epithelial cell injury and its underlying mechanism of action and development of new drugs against it are crucial steps to prevent and treat respiratory diseases. Hydrogen peroxide (H₂O₂) is the main reactive oxygen species (ROS) involved in the regulation of redox reactions in biological activities through specific protein targets [1]. Recently, oxidative stress is considered as a key risk factor for respiratory diseases [2]; evidences showed that the causative factors of diseases including mycoplasma pneumonia are also linked with the oxidative stress [3–6]. Therefore, the active compounds with antioxidative properties tend to be the potential agents for the prevention of respiratory diseases.

In recent years, the use of active natural compounds against pathological conditions has gained considerable recognition. 4,7-Didehydro-neophysalin B and Physalin B are biologically active substances extracted from Physalis alkekengi L. var. franchetti. It has been confirmed that Physalin B has various pharmacological effects such as anti-inflammatory [7], antitumor [8], antibacterial [9], and immune regulation [10] verified by various bioassay both
Table 1: Nrf2 siRNA: a pool of 3 different siRNA duplexes.

| siRNA | Primer (5′→3′)                     |
|-------|-----------------------------------|
| Nrf2 A | Sense: GCAUGCUACUGUGAUGAAGAtt, Anti-sense: UCUUCAUACCGUAGACUGCtt |
| Nrf2 B | Sense: CUCUCAUGUGUAGUGAAAtt, Anti-sense: UUUCACAUACAGUAGGAtt |
| Nrf2 C | Sense: GUGUCAGAUGUAGUAUCAtt, Anti-sense: UGAUACACAUAGCGACAtt |

Table 2: Primers used in RT-PCR.

| Gene | Primer (5′→3′)                     |
|------|-----------------------------------|
| Nrf2 | Forward: GAGAGCCCGATCTTTGATTGC, Reverse: TGGGATCTTGGACTTTGAAC |
| Keap1 | Forward: TTCAAGGCACTGTTCACCAAA, Reverse: TGAGTACCCCTTATGGACACC |
| NQO1 | Forward: AGTTGGGTAGTGAAGACCTGCCTTC, Reverse: CCGCTTCTCGTCACAACAT |
| HO-1 | Forward: TGTGTTCCTCTGCTGACATAC, Reverse: GGGAAAACCTGCGAAAA |
| KLF9 | Forward: CGTTCACCTGTATGCACTGTA, Reverse: GAGATTTACTGCGCTGCTC |
| GAPDH | Forward: GAGATTTACTGCGCTGCTC, Reverse: GACTCATGCTACCTCT |

RT-PCR was performed on the ABI PRISM® 7500 real-time PCR analyzer (Applied Biosystems, Foster City, CA, USA) using the SYBR® Premix Ex Taq™ RT-PCR Kit. The relative mRNA expression was calculated by means of 2^−ΔΔCt and was normalized to the mean mRNA expressions of GAPDH. The results were calculated with the following formula: ratio = 2^−ΔΔCt, ΔΔCt = (Ct_target − Ct_GAPDH)Sample − (Ct_target − Ct_GAPDH)Control.

in vitro and in vivo. 4,7-Didehydro-neophysalin B is a new kind of Physalin B lacking two hydrogen atoms. There is evidence that Physalin B has efficient antioxidant activity [8]. However, the protective effect and mechanism of 4,7-didehydro-neophysalin B against H2O2-induced lung injury remained elusive.

Table 2: Primers used in RT-PCR.

| Gene | Primer (5′→3′)                     |
|------|-----------------------------------|
| Nrf2 | Forward: GAGAGCCCGATCTTTGATTGC, Reverse: TGGGATCTTGGACTTTGAAC |
| Keap1 | Forward: TTCAAGGCACTGTTCACCAAA, Reverse: TGAGTACCCCTTATGGACACC |
| NQO1 | Forward: AGTTGGGTAGTGAAGACCTGCCTTC, Reverse: CCGCTTCTCGTCACAACAT |
| HO-1 | Forward: TGTGTTCCTCTGCTGACATAC, Reverse: GGGAAAACCTGCGAAAA |
| KLF9 | Forward: CGTTCACCTGTATGCACTGTA, Reverse: GAGATTTACTGCGCTGCTC |
| GAPDH | Forward: GAGATTTACTGCGCTGCTC, Reverse: GACTCATGCTACCTCT |

RT-PCR was performed on the ABI PRISM® 7500 real-time PCR analyzer (Applied Biosystems, Foster City, CA, USA) using the SYBR® Premix Ex Taq™ RT-PCR Kit. The relative mRNA expression was calculated by means of 2^−ΔΔCt and was normalized to the mean mRNA expressions of GAPDH. The results were calculated with the following formula: ratio = 2^−ΔΔCt, ΔΔCt = (Ct_target − Ct_GAPDH)Sample − (Ct_target − Ct_GAPDH)Control.

In this study, H2O2 was used to establish the basic oxidative damage model so that we can investigate the effect of 4,7-didehydro-neophysalin B on the oxidative stress, cell apoptosis, and the role of Nrf2 signaling pathway. This study will promote the development of nutraceutical and functional food from Physalis alkekengi L. var. franchetii or its extracts for reducing the risk of oxidative stress-induced lung injury.

2. Materials and Methods

2.1. Preparation of 4,7-Didehydro-neophysalin B. Physalin was isolated from the stems of Physalis alkekengi L. var. franchetii. The plant was identified by Dr. Qiongming Xu from the College of Pharmaceutical Science, Soochow University. Physalin was purified, and the purity of Physalin is 72.39% by chromatography [17]. Physalin from the above reaction was pulverized, extracted with ethanol, loaded on a silica gel column, eluted, and purified with ethyl acetate. The obtained contents of 4,7-didehydro-neophysalin B were detected by chromatography.

2.2. Cytotoxicity of 4,7-Didehydro-neophysalin B. The RLE-6TN cells (ATCC® RLE-2300™, 4000 cells per well) were subcultured in 96-well plates. After different concentrations of 4,7-didehydro-neophysalin B (50, 25, 10, and 5 μg/mL) treatments, cells were incubated with CCK-8 (CK04, Dojindo, Japan) solution (10%) for 1 h at 37°C. The resulting absorbance was measured at 450 nm by using a microplate reader (Tecan, Switzerland). The maximum nontoxic concentration (MNTC) value was calculated by Prism 6 software (San Diego, CA, USA) [18].

Cell proliferation rate = \frac{A_{450} \text{ of treatment group}}{A_{450} \text{ of blank group}} \times 100\%.

2.3. Culture of RLE-6TN Cells. The RLE-6TN cell line was cultured in DMEM containing 15% fetal bovine serum, 2 mmol/L glutamine, 5 mmol/L sodium pyruvate, and 25 mmol/L HEPES, containing 100 U/mL penicillin and 100 μg/mL streptomycin, placed in an incubator and grown at 37°C, 5% CO2-saturated humidity. 0.25% trypsin-EDTA digested and passaged, and cells in logarithmic growth phase were used for experiments [19]. RLE-6TN cells were seeded in 96-well cell culture plates and incubated in a 5% CO2 humidified incubator for 24 h at 37°C. Various concentrations of H2O2, prepared in 2% medium (200, 100, 50, and 25 μmol) were added and incubated again for 4, 8, 12, and 24 h. The cell proliferation rate of H2O2 was determined using the CCK-8 (CK04, Dojindo, Japan) kit. To develop a cellular oxidative damage model, H2O2 (100 μmol) prepared in 2% medium was added to each well except the blank group and incubated for the next 12 h. Different concentrations of 4,7-didehydro-neophysalin B with low-dose group (2.5 μg/mL), medium-dose group (5 μg/mL), and high-dose
group (10 μg/mL) were added as treatment and incubated again for 24 h.

Cell proliferation rate = \( \frac{A_{450} \text{ of treatment group}}{A_{450} \text{ of blank group}} \times 100\% \) 

2.4. Transfection. (1) Culture the RLE-6TN cells for 3 to 5 generations, select the cells with good growth condition, digest the cells with 0.25% trypsin, inoculate \( 2 \times 10^5 \) cells/well in 6-well plates, add 10% fetal bovine serum, culture in F12 medium (Gibco, USA) without double antibody for 24 h, and after the cell confluence reached 70%, transfection was carried out. (2) Mix 7.5 μL of Lipofectamine 2000 (Life, USA) with 125 μL of OPTI-MEM (Gibco, USA) medium. Take another EP tube and mix 6 μL of siRNA (0.75 μg) with 125 μL of OPTI-MEM medium. This is the amount for one well in a 6-well plate. (3) Gently mix the two tubes of mixture in step 2, and let them act together at room temperature for 15 min. (4) The liquid after the joint action in step 3 was directly added to the original 6-well plate medium, and after 6 hours in a 37°C, 5% CO₂ incubator, the medium was replaced and the subsequent experiments were carried out. For siRNA transfection, siRNA duplex targeting Nrf2 (sc-37030, Santa Cruz Biotechnology, Santa Cruz, CA, USA, primer shown in Table 1) were used. siRNA (sc-37007, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was selected as a
control to determine whether Nrf2’s siRNA was successfully transfected.

2.5. Detection of Gene Expression in Tissues by RT-PCR. The total RNA was extracted from cells using TRIzol reagent, and the absorbance was measured at 260 and 280 nm by an ultraviolet spectrophotometer. The RNA content was calculated from the absorbance (A) at a wavelength of 260 nm while the RNA purity was identified by the ratio of $A_{260}/A_{280}$. Reverse transcription of total RNA was performed with reverse transcriptase. The PCR primers (shown in Table 2) were designed and synthesized by KeyGEN BIO-TECH with GAPDH as endogenous control, and the PCR reactions were carried out according to the recommended conditions.

2.6. Flow Cytometry. After treating the cells with/without 100 μmol of H$_2$O$_2$ for 12 h, different concentrations of 4,7-didehydro-neophysalin B (0, 2.5, 5, and 10 μg/mL) were added to the rat lung epithelial cells RLE-6TN for 24 h. The cells were collected, washed twice with cold PBS, and centrifuged at 1000 rpm for 5 min, the supernatant was discarded, and the cells were made into a single cell suspension. 5 μL of Annexin V-FITC and 5 μL PI were added, mixed, and incubated for 15 min in the dark. Finally, 400 μL of binding buffer was added and cell apoptosis was detected using flow cytometry.

2.7. Western Blot. RLE-6TN cells were homogenized with RIPA lystate (Solarbio, Beijing, China) containing protease inhibitor PMSF and phosphatase inhibitor (Solarbio). The nuclear and cytosolic proteins were extracted by a nuclear protein extraction kit (KeyGEN BioTECH, Jiangau, China). The protein concentrations were measured by bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China). 40 μg equivalent protein samples were separated by SDS-PAGE gel electrophoresis and were transferred into a polyvinylidene fluoride (PVDF) membrane. The membranes were incubated with appropriate primary and HRP-conjugated secondary antibodies. Chemiluminescence was visualized with an enhanced chemiluminescence kit (BOSTER, Wuhan, China). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein (Sanying, Wuhan, China) and the TATA-binding protein (TPB) (Sanying, Wuhan, China) were used as loading control. The antibodies for Nrf2, heme oxygenase-1 (HO-1), nicotinamide adenine dinucleotide phosphatase-quinone-acceptor 1 (NQO1), Krueppel-like factor 9 (KLF9), Keap-1, p53, Bcl-2-associated X protein (Bax), B cell lymphoma gene 2 (Bcl-2), and B cell lymphoma-extra large (Bcl-xL) were purchased from Abcam (UK). All secondary antibodies were from Sanying (Wuhan, China). Protein expression was analyzed by scanning densitometry using ImageJ software (USA).

To further explain the molecular mechanism by which 4,7-didehydro-neophysalin B treated H$_2$O$_2$-induced oxidative damage in RLE-6TN cells, western blot was performed. Nrf2’s siRNA was generated to inhibit Nrf2 expression, and the inhibitory efficiency was verified using western blot. Nrf2’s siRNA was transfected into RLE-6TN cells with or without H$_2$O$_2$ treatment. Then, 10 μg/mL 4,7-didehydro-neophysalin B was administrated for treatment and the expression of Nrf2 was explored using western blotting.

2.8. Statistical Analysis. Statistical analysis was performed by one-way analysis of variance with Tukey’s test post hoc comparisons and Student’s t-test when comparing between 2 groups using SPSS 19.0 software (USA). The data were presented as the mean ± SEM. Values with $p < 0.05$ was considered statistically significant.

3. Results

3.1. Structure and Content of 4,7-Didehydro-neophysalin B. The structure and contents of 4,7-didehydro-neophysalin B are shown in Figures 1(a) and 1(b). The chemical formula of 4,7-didehydro-neophysalin B is C$_{28}$H$_{28}$O$_{9}$, and its purity is 99.01%.

3.2. The Cytotoxicity of 4,7-Didehydro-neophysalin B. The maximum nontoxic concentration of 4,7-didehydro-neophysalin B in RLE-6TN was investigated. Compared with the blank group, RLE-6TN cells treated with 25 μg/mL 4,7-didehydro-neophysalin B for 24 h showed significant reduction in cell viability (74.87 ± 1.54%, $p = 0.008 < 0.01$) while 10 μg/mL 4,7-didehydro-neophysalin B had negligible effect on cell viability (93.6 ± 1.03%, $p = 0.07 > 0.05$) (Figure 2). Therefore, we choose 10 μg/mL 4,7-didehydro-neophysalin B as the maximum nontoxic concentration.

3.3. The Effect of 4,7-Didehydro-neophysalin B on RLE-6TN Pretreated with H$_2$O$_2$. Compared with the blank group, the working concentration for H$_2$O$_2$ in RLE-6TN cells treated with different concentrations was determined. Results showed that 100 μmol H$_2$O$_2$ treated for 12 h significantly reduced (59.87 ± 1.32%, $p = 0.0004 < 0.001$) while 50 μmol H$_2$O$_2$ showed less effect on the cell viability (80.37 ± 1.96%, $p = 0.0007 < 0.001$). The mortality of cells treated with 200 μmol of H$_2$O$_2$ was too high (41.28 ± 2.84%, $p = 0.0003$).
Therefore, 100 μmol H2O2 was selected as a working concentration. Compared with the blank group, the working time for 100 μmol H2O2 in RLE-6TN cells treated with different times was determined. The results showed that RLE-6TN cells treated with 100 μmol H2O2 for 24, 12, 8, and 4 h treatment all had significant effect on cell viability, but the RLE-6TN cells treated with 100 μmol H2O2 for 24 (37.84 ± 1.62%, p = 0.0005 < 0.001), 8 (76.62 ± 1.45%, p = 0.0006 < 0.001), and 4 (87.61 ± 2.33%, p = 0.0009 < 0.001) hours were too high or low on cell viability, as shown in Figure 3(b). Therefore, 12 h (68.88 ± 0.95%, p = 0.0002 < 0.001) was selected as a working time.

Conclusively, 100 μmol of H2O2 was used to treat RLE-6TN for 12 h, followed by the addition of 4,7-didehydro-neophysalin B to examine the effect on cell viability. As shown in Figure 3(c), 4,7-didehydro-neophysalin B had significantly reversed the cell viability caused by H2O2 in a dose-dependent manner in which 10 μg/mL 4,7-didehydro-neophysalin B showed the strongest effect (84.93 ± 1.86%, p = 0.0007 < 0.001).

3.4. Expressions of Nrf2 in RLE-6TN under the Intervention of Nrf2’s siRNA

The mRNA expression of Nrf2 and its downstream genes was detected by RT-PCR. The A260/A280 ratio of the RNA samples was 1.8-2.0. Nrf2’s siRNA treatment significantly reduced the mRNA expression of Nrf2 and its downstream genes compared with the control siRNA.
which have shown negligible effect on the mRNA expression of Nrf2 and its downstream genes, as shown in Figure 4.

3.5. 4,7-Didehydro-neophysalin B Mitigates H₂O₂-Induced Apoptosis in RLE-6TN. We investigated the effect of 4,7-didehydro-neophysalin B on H₂O₂-induced cell apoptosis by flow cytometry. Compared with the blank group, H₂O₂ caused a significant increase in RLE-6TN cell apoptosis while only 4,7-didehydro-neophysalin B treatment showed no significant effect on cell viability. Similarly, 4,7-didehydro-neophysalin B treatment dramatically mitigated H₂O₂-induced apoptosis. These results elucidated that 4,7-didehydro-neophysalin B had only a significant effect on H₂O₂-induced early apoptosis but had no significant effect on late apoptosis (early apoptosis: 4,7-didehydro-neophysalin B group 7.98 ± 1.15%, \( p = 0.07 > 0.05 \); model group 37.48 ± 3.84%, \( p = 0.0005 < 0.001 \); low-dose group 29.83 ± 3.68%, \( p = 0.0007 < 0.001 \); medium-dose group 21.44 ± 2.99%, \( p = \)
Figure 6: Western blot analysis of the Nrf2 signaling pathway. (a) After being added with different concentrations of 4,7-didehydro-neophysalin B, Nrf2 pathway-related protein expression in RLE-6TN cells and its quantitative band intensity analysis. (b) Effect of 4,7-didehydro-neophysalin B on the expression of Nrf2 pathway-related proteins and the quantitative band intensity analysis. (c) Expression of Nrf2 protein in the nucleus and its quantitative band intensity analysis. Compared with the blank group, \(^* p < 0.05\), \(^{**} p < 0.01\), and \(^{***} p < 0.001\). Compared with the H\(_2\)O\(_2\)+0% 4,7-didehydro-neophysalin B group, \(^{#} p < 0.05\), \(^{##} p < 0.01\), and \(^{###} p < 0.001\).
0.0008 < 0.001; high-dose group 15.35 ± 1.68%, \( p = 0.03 < 0.05 \), compared with the blank group; model group 24.49 ± 2.96%, \( p = 0.006 < 0.01 \); low-dose group 17.92 ± 2.86%, \( p = 0.007 < 0.01 \); medium-dose group 15.58 ± 3.15%, \( p = 0.008 < 0.01 \); high-dose group 19.44 ± 3.57%, \( p = 0.006 < 0.01 \), compared with the blank group), as shown in Figure 5(a). The microscopic images of cells after treatment are shown in Figure 5(b).

3.6. The Effect of 4,7-Didehydro-neophysalin B on Nrf2-Dependent Signaling Pathway in \( \text{H}_2\text{O}_2 \)-Induced Oxidative Damage. Nrf2 signaling pathway proteins were detected by western blotting. In vitro treatment of \( \text{H}_2\text{O}_2 \) significantly reduced the Nrf2, NQO1, Keap1, HO-1, and KLF9 protein expressions. However, 4,7-didehydro-neophysalin B treatment has significantly reversed these effects induced by \( \text{H}_2\text{O}_2 \) with 10 \( \mu \text{g/mL} \) 4,7-didehydro-neophysalin B showing the strongest effect as shown in Figure 6(a). The addition of 4,7-didehydro-neophysalin B alone showed no effect on the protein expression of Nrf2, NQO1, Keap1, HO-1, and KLF9 in RLE-6TN as shown in Figure 6(b). \( \text{H}_2\text{O}_2 \) had markedly reduced the expression of nuclear Nrf2 which was significantly reversed by 4,7-didehydro-neophysalin B as shown in Figure 6(c).

3.7. Knockdown of Nrf2 Declines the Treatment Effect of Physalin B. The Nrf2's siRNA treatment significantly reduced the expression of Nrf2 as shown in Figure 7. Compared with the model group, the treatment effect of 4,7-didehydro-neophysalin B in the Nrf2's siRNA treatment group was significantly reduced.

3.8. Effects of 4,7-Didehydro-neophysalin B on Bcl-2 Family and p53 Proteins. The molecular basis of a protective effect of 4,7-didehydro-neophysalin B against \( \text{H}_2\text{O}_2 \)-induced cell apoptosis was investigated. \( \text{H}_2\text{O}_2 \) treatment decreased the expression of antiapoptotic Bcl-2 and Bcl-xL proteins in RLE-6TN cells. In \( \text{H}_2\text{O}_2 \)-treated cells, 4,7-didehydro-neophysalin B administration significantly induced the expression of Bcl-2 and Bcl-xL. The expression of proapoptotic protein Bax was significantly increased post \( \text{H}_2\text{O}_2 \) treatment which was significantly reduced by 4,7-didehydro-neophysalin B treatment. Compared with the control group, p53 expression was also increased post \( \text{H}_2\text{O}_2 \) treatment. 4,7-Didehydro-neophysalin B attenuated \( \text{H}_2\text{O}_2 \)-induced p53 expression with the strongest effect of 10 \( \mu \text{g/mL} \) 4,7-didehydro-neophysalin B as shown in Figure 8(a) (Bcl-xL expression: blank 1.0 ± 0.14, control 0.37 ± 0.05, \( p = 0.004 < 0.01 \) compared with the blank group; low 0.28 ± 0.07, mid 0.59 ± 0.09, \( p = 0.03 < 0.05 \) compared with the control group; high group 0.98 ± 0.05, \( p = 0.11 \) compared with the control group; Bcl-2 expression: blank 1.0 ± 0.16, control 0.48 ± 0.35, \( p = 0.005 < 0.01 \) compared with the model group; low 0.53 ± 0.005, mid 0.59 ± 0.77, \( p = 0.04 < 0.05 \) compared with the control group; high group 0.98 ± 0.05, \( p = 0.11 < 0.01 \) compared with the control group). The addition of 4,7-didehydro-neophysalin B alone showed no effect on the protein expression of Bcl-2, Bcl-xL, Bax, and p53 in RLE-6TN as shown in Figure 8(b).

4. Discussion

A variety of chemicals and environmental factors causes oxidative stress in the human body which damages and induces a variety of diseases. \( \text{H}_2\text{O}_2 \)-induced RLE-6TN cell damage is a classical model of oxidative stress injury. Studies have shown that the damage caused by oxidative stress is mediated by reactive oxygen species which plays an important role in neurodegenerative diseases [20, 21]. \( \text{H}_2\text{O}_2 \) produced in the body during metabolism is equivalent to reactive oxygen species and can aggravate the body oxidative stress injury [22, 23]. The results of this study showed that \( \text{H}_2\text{O}_2 \) induced oxidative damage and apoptosis in RLE-6TN cells which was reduced by 4,7-didehydro-neophysalin B treatment as shown in Figure 2(c). This study demonstrated that
4,7-didehydro-neophysalin B possessed therapeutic effects in the form of antioxidant activities against H$_2$O$_2$-induced oxidative stress injury.

When the cellular level of ROS exceeds the body's antioxidant capacity, it induces oxidative stress leaving the cells in a redox state by producing peroxides and free radicals [24–26]. Mitochondrial dysfunction and DNA damage are caused by the accumulation of reactive oxygen species which mediate and accelerate apoptosis [27–30]. DNA damage causes activation of p53 which serves as a major mediator of cellular stress [31]. Our results (Figure 7) indicated p53-mediated apoptosis in H$_2$O$_2$-induced lung injury. p53 along with the members of Bcl-2 family proteins regulates protein-protein interactions and causes activation of Bax which promotes mitochondrial membrane permeability and hence induces apoptosis [32]. In this study, 4,7-didehydro-neophysalin B suppressed p53, decreased the level of proapoptotic member Bax, and increased the level of prosurvival members Bcl-2 and Bcl-xL. Moreover, the results of apoptotic index (Figure 2) and cell viability (Figure 4) of RLE-6TN were consistent with the observed effects on Bcl-2 family proteins. These findings suggest that 4,7-didehydro-neophysalin B has a pivotal role in regulating apoptosis and inhibiting oxidative stress-induced cell death. It is also noteworthy that some studies showed that NQO1 stabilizes the tumor suppressor p53 [33]. HO-1 upregulates Bcl-2 and Bcl-xL expressions [34] which are downstream proteins of Nrf2. A possible explanation is that 4,7-didehydro-neophysalin B protects lung injury induced by H$_2$O$_2$ via activating the Nrf2 pathway.

The transcription factor Nrf2 plays an important role in protection against oxidative damage [35, 36]. Nrf2 senses the presence of oxidative stress and regulates transcription of genes encoding cytoprotective enzymes and other proteins crucial for maintaining cellular homeostasis. Under physiological conditions, the Nrf2 inhibitor, Keap-1, is a
5. Conclusions

In conclusion, our study demonstrated that Physalin B attenuated H₂O₂-induced lung injury by regulating the Nrf2/P53 signaling pathway. Physalin B with great therapeutic potential can be used as an antioxidant agent. This study provided beneficial evidences for the application of Physalin B supplementation as an alternative treatment strategy for lung injury.

Abbreviations

H₂O₂: Hydrogen peroxide  
ROS: Reactive oxygen species  
Nrf2: Nuclear translocation of erythroid-2-related factor 2  
PVDF: Polyvinylidene fluoride  
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase  
ARE: Antioxidant response element  
TBP: TATA-binding protein  
HO-1: Heme oxygenase-1  
NQO1: Nicotinamide adenine dinucleotide phospha- sequinone-acceptor 1  
KLF9: Krueppel-like factor 9  
Keap-1: Kelch-like ECH-associated protein  
Bax: Bcl-2-associated X protein  
Bcl-2: B cell lymphoma gene 2  
Bcl-xL: B cell lymphoma-extra large  
SEM: Standard errors of the mean.

Data Availability

The original contributions presented in the study are included in the article; further inquiries can be directed to the corresponding author.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Authors’ Contributions

Conceptualization and methodology were done by Qiu Zhong and Yao-gui Sun; investigation, data curation, and writing—original draft preparation were done by Qiu Zhong; validation was done by Qiu Zhong and Yao-gui Sun; writing—review and editing were done by Ajab Khan, Jiahua Guo, Zhirui Wang, and Na Sun; funding acquisition was done by Yaogui Sun and Hongquan Li; project administration was done by Hongquan Li. All authors have read and agreed to the reviewed version of the manuscript.

Acknowledgments

This work was supported by the Key Research and Development Plan of Shanxi Province (grant numbers 202102140601019 and 201803D221023-3).

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