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간세포에서 산화적 스트레스 억제를 통한 생달가지 추출물의 세포보호 효과

양지혜*

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Cytoprotective Effect of *Cinnamomum japonicum* Siebold Branch Extracts via Blocking Oxidative Stress in Hepatocytes

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ABSTRACT

Objectives : Native to East Asia, Japan, and Korea, *Cinnamomum japonicum* Siebold (CJ) is renowned for its aromatic leaves and bark. We previously assessed the antioxidant activity of fractionated CJ branches (CJB:70% EtOH extract), including hexane (CJB1), chloroform (CJB2), ethyl acetate (CJB3), butanol (CJB4), and water (CJB5). Our findings revealed that CJB3 exhibited the highest antioxidant activity. Here, we aimed to investigate whether CJB3 possesses cytoprotective effects and induces the activity of antioxidant enzymes in hepatocytes.

Methods : As HepG2 cells were the first to exhibit the key characteristics of hepatocytes, we investigated the hepatoprotective effects of CJB3 on HepG2 cells.

Results : Before conducting the cell experiment, we checked that CJB3, up to a concentration of 100 μg/mL, did not exhibit cytotoxicity toward HepG2 cells. ROS production increased because of t-BHP treatment decreased in a concentration-dependent manner upon CJB3 treatment. We confirmed that CJB3 inhibited t-BHP-induced cell death. CJB3 was found to reverse the expression of proteins associated with t-BHP-induced apoptosis. We also observed that CJB3 induced Nrf2 phosphorylation and the nuclear translocation of Nrf2. And, CJB3 treatment caused a time-dependent enhancement of GCL and NQO1 protein expression. We further confirmed that CJB3 increased the expression of Nrf2 target genes, and this effect was associated with the activation of JNK, p38, and AMPK.

Conclusion : CJB3 prevents t-BHP-induced oxidative stress and apoptosis and enhances the expression of Nrf2 target genes via JNK, p38, and AMPK activation. These results suggest that CJB3 is a promising candidate for the treatment of liver diseases.

Key-words : *Cinnamomum japonicum* Siebold Branch, Hepatocyte, Oxidative stress, Apoptosis, Nrf2

1. Introduction

Hepatocytes, constituting around 80% of the liver mass, serve as the primary functional cells in the liver and play a central role in its diverse functions[1,2]. These vital cells are responsible for numerous processes, including carbohydrate, lipid, and protein metabolism, detoxification, and immune cell activation, all essential for maintaining liver homeostasis[3,4]. Any damage to hepatocytes, caused by various factors, is a crucial factor in the development and progression of liver diseases[5]. Elevated oxidative stress can harm hepatocytes, leading to cell death through apoptosis. Reactive oxygen species (ROS) can damage mitochondrial components, causing mitochondrial dysfunction, which, in turn, triggers the release of pro-apoptotic proteins like cytochrome c. Additionally, ROS can directly activate caspases, the key enzymes involved in apoptosis[6,7].

Antioxidant enzymes play a pivotal role in neutralizing ROS and safeguarding cells from oxidative damage. Glutamate-cysteine ligase (GCL), also known as gamma-glutamylcysteine synthetase, stands as the initial rate-limiting enzyme in glutathione synthesis and holds significance as an antioxidant and cellular protectant[8]. NADPH Quinone Oxidoreductase 1 (NQO1) is instrumental in detoxifying highly reactive and potentially harmful quinones. By reducing quinone levels, NQO1 actively participates in cellular antioxidant defense, preventing the formation of reactive intermediates that could cause oxidative stress and damage cellular components[9]. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor central to the regulation of antioxidant enzymes. Serving as a master regulator, Nrf2 orchestrates the cellular defense against oxidative stress by governing the expression of genes involved in antioxidant pathways[10,11]. Thus, Nrf2 activation can be considered a therapeutic target for conditions associated with oxidative stress and certain diseases such as liver disease.

Cinnamomum japonicum Siebold (CJ), commonly referred to as Japanese cinnamon or Japanese camphor tree, is an evergreen tree or large shrub belonging to the Lauraceae family. Native to East Asia, Japan, and Korea, CJ is renowned for its aromatic leaves and bark[12]. Modern pharmacological studies have highlighted various biological activities associated with CJ. In a previous study, CJ branches (CJB:70% EtOH extract) using hexane (CJB1), chloroform (CJB2), ethyl acetate (CJB3), butanol (CJB4), and water (CJB5). Their antioxidative activities and phenolic contents were measured, revealing that CJB3 exhibited the highest antioxidative activity as well as flavonoid and phenolic content among the fractions. Additionally, we have explored the anti-inflammatory efficacy of CJB3 and elucidated its mechanism of action[13]. However, the antioxidant and protective effects of CJB3 in cells, along with the underlying mechanisms, have not been investigated thus far. Hence, the current study aimed to determine whether CJB3 could attenuate t-BHP-induced ROS production and cell death in hepatocytes and whether it could induce antioxidant enzymes and Nrf2 activation.

2. Materials and Methods

2.1. Materials

MTT, DCFH-DA, and Compound C (AMPK inhibitor) were sourced from Sigma Chemicals (St. Louis, MO, USA). PD98059 (an ERK inhibitor), SB 203580 (a p38 inhibitor), and SP600125 (a

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JNK inhibitor) were procured from Millipore (Bedford, MA, USA). Antibodies against Nrf2, Bax, Bcl-xL, and PARP were obtained from Santa Cruz Biotechnology (Dallas, TX, U.S.A.). p-Nrf2 (Ser40) antibody was obtained from Novus (EP1809Y). Antibodies against NQO1, caspase-3 and lamin A/C were purchased from Cell Signaling Technology (Danvers, MA, U.S.A.). GCL antibody was purchased from Abcam (Cambridge, MA, U.S.A.). β-actin antibody was sourced from Sigma Chemicals (St. Louis, MO, U.S.A.).

2.2. Preparation of CJB3

CJB3 was prepared as described previously[13]. The summary is as follows: First, the fresh *Cinnamomum japonicum* Siebold branches were dried at 40° C. The dried branches (100 g) were then powdered and extracted. Next, CJ branches were extracted using 70% EtOH for 7 days at room temperature (25 ± 2 ° C). The 70% EtOH extract was separated using hexane, chloroform, ethyl acetate (CJB3), butanol, and water. Finally, CJB3 was vacuum concentrated.

2.3. Cell Culture

HepG2 cells were procured from the American Type Culture Collection (ATC C, Manassas, VA, U.S.A.) and cultured in DMEM supplemented with 10% fetal bovine serum and 50 units/mL penicillin/streptomycin. The cells were maintained at 37° C in a humidified 5% CO_2 atmosphere.

2.4. MTT Assay

For cell viability assessment, cells were seeded in 48 well plates and treated with t-BHP and/or CJB3. Subsequently, viable cells were stained with 0.2 mg/mL MTT for 1 h. Afterward, the media were removed, and formazan crystals formed in the wells were dissolved using 200 μL of dimethyl sulfoxide. The absorbance was measured at 540 nm using a microplate reader (Spectramax; Molecular Devices, Sunnyvale, CA, USA).

2.5. Measurement of ROS Generation

DCFH-DA is a cell-permeable, non-fluorescent probe that undergoes cleavage by intracellular esterases, converting it into highly fluorescent dichlorofluorescein upon reaction with H_2O_2. Following treatment with t-BHP (500 μM, 1 h), cells were stained with 10 μM DCFH-DA for 30 min at 37° C. The generation of H_2O_2 was assessed by measuring the fluorescence intensity of dichlorofluorescein using a fluorescence microplate reader (Jemini; Molecular Devices) with excitation and emission wavelengths of 485 and 530 nm, respectively.

2.6. Immunoblotting

Protein extraction, subcellular fractionation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblotting were conducted following previously described methods[14]. In brief, samples were separated on a 7.5% polyacrylamide gel and then transferred onto nitrocellulose membranes. The membranes were incubated with specific primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies. Immunoreactive proteins were detected using an ECL chemiluminescence detection kit (Amersham Biosciences). Equal protein loading and the integrity of subcellular fractionation were confirmed by examining β-actin expression in the immunoblots.

2.7. Statistical Analysis

The statistical significance of differences among groups was assessed using one-way analysis of variance (ANOVA) For each statistically significant treatment effect, the Newman-Keuls test was employed for comparisons between multiple groups. The data were presented as means ± standard error (S.E.).

3. Results

3.1. Evaluation of cytotoxicity of CJB3 in HepG2 Cell

Hepatocyte damage is a common occurrence in

liver injuries and significantly contributes to the development of conditions such as hepatitis, liver fibrogenesis, and cirrhosis[15]. Among hepatic cell lines, HepG2 cells were the first to demonstrate essential hepatocyte characteristics[16]. Therefore, our study focused on investigating the hepatoprotective effects of CJB3 on HepG2 cells. Before conducting the cell experiment, we performed an MTT assay to establish the optimal concentration of CJB3 for treatment. HepG2 cells were treated with varying concentrations of CJB3(10-100 ㎍/mL) for 24 h. The MTT assay results indicated that CJB3, even at a concentration of 100 μg/mL, did not exhibit cytotoxicity against HepG2 cells (Figure 1).

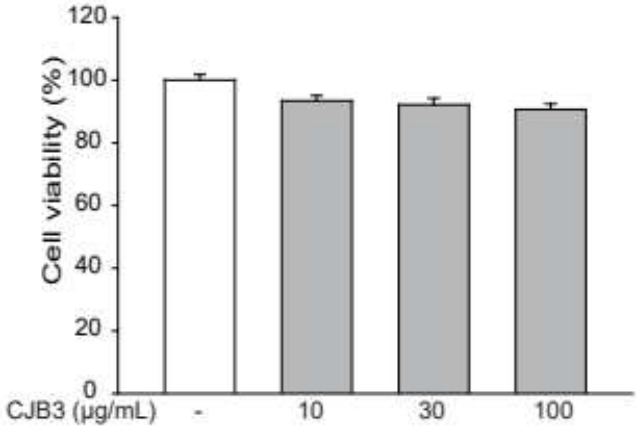


Figure 1. The cytotoxicity measurement of CJB3 in HepG2 cells using MTT assay. CJB3 was treated at concentrations from 10 ㎍/mL to 100 ㎍/mL, and incubated for 24 h. Data represents the mean ± S.E. of three replicates (compared to vehicle-treated control).

3.2. CJB3 inhibits t-BHP induced-Oxidative Stress in HepG2 Cell

ROS are oxygen-containing molecules with the potential to harm cells[17,18]. An imbalance and excessive production of ROS can result in oxidative stress, causing damage to cells. Tert-butyl hydroperoxide (t-BHP) is a well-known inducer of oxidative stress and cellular damage, acting, in part, by depleting the antioxidant glutathione. In this study, we utilized t-BHP to induce intracellular ROS production[19]. HepG2 cells were pre-treated with CJB3 for 3 h and then exposed to t-BHP for 1h. The production of ROS was elevated by t-BHP and subsequently reduced by CJB3 treatment (Figure 2). These results demonstrate that CJB3 effectively inhibited t-BHP-induced oxidative stress in HepG2 cells.

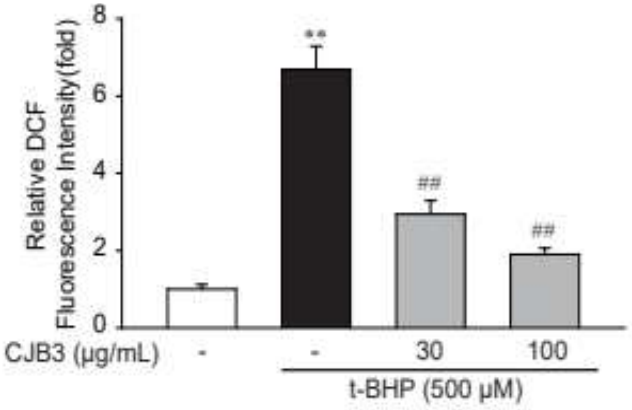
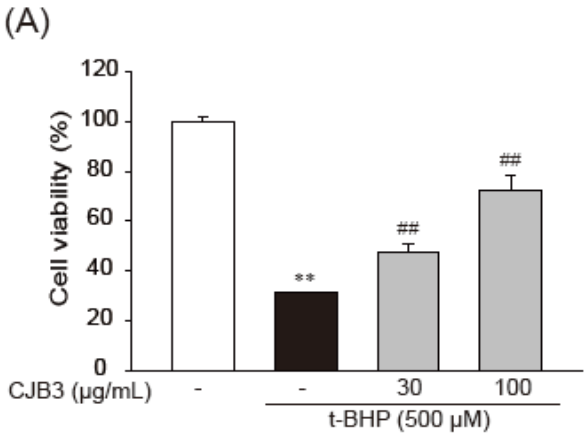


Figure 2. The measurement of ROS production using DCFH-DA. CJB3 was pre-treated at concentrations from 30 μg/mL to 100 μg/mL for 3 h, and then t-BHP (500 μM) was treated for 1 h. Data represents the mean ± S.E. of three replicates; **P<0.01 significant versus vehicle-treated control; ##P<0.01, significant versus t-BHP alone.

3.3. CJB3 suppresses t-BHP induced-Cell Death in HepG2 Cell

ROS can function as signaling molecules in the regulation of apoptosis[7]. Elevated ROS levels can inflict damage on cellular structures and DNA, leading to apoptosis[6]. In this study, we investigated the protective effects of CJB3 against excessive oxidative stress-induced cell death. HepG2 cells pre-treated with CJB3 for 3 h were exposed to t-BHP (500 μM, 6 h) to induce cell death. The results from the MTT assay affirmed that CJB3 effectively inhibited cell death caused by t-BHP (Figure 3 A). Furthermore, CJB3 reversed the expression of proteins involved in t-BHP-induced apoptosis (Figure 3B). These findings strongly indicate that CJB3 suppressed t-BHP-induced cell death in HepG2 cells.



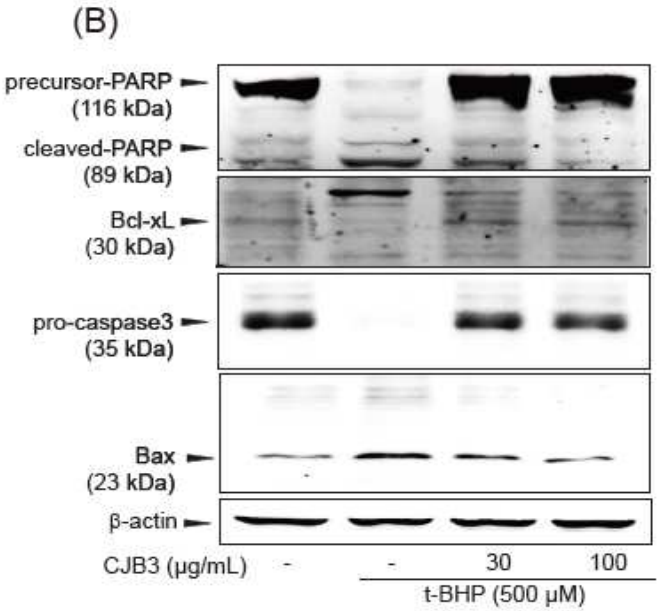


Figure 3. The cytoprotective efficacy of CJB3 in HepG2 cells. (A) The efficacy of CJB3 on t-BHP-induced cell death. HepG2 cells were treated with t-BHP (500 μM) and/or 30-100 μg/mL CJB3 for 3 h. Cell viability was assessed using an MTT assay. Data represent the mean ± standard error (S.E.) of three experiments; **p < 0.01 vs. vehicle-treated control; ##p < 0.01 vs. t-BHP treatment alone. (B) Representative western blots analyzing the levels of proteins involved in cell death in the presence or absence of CJB3 and/or t-BHP. The levels of marker proteins (precursor-PARP, cleaved-PARP, Bcl-xL, pro-caspase3, and Bax) of apoptosis in HepG2 cell lysates as determined by immunoblotting. The experiments were repeated thrice.

3.4. CJB3 induces Antioxidant Enzymes in HepG2 Cell

Antioxidant enzymes serve as guardians of cellular health, tirelessly working to neutralize[20]. ROS and prevent oxidative stress. The typical antioxidant enzymes include GCL and NQO1. GCL, an important antioxidant, is a key enzyme involved in glutathione synthesis. NQO1 is involved in the detoxification of quinones, which can be harmful because of their ability to generate ROS[21]. We investigated whether CJB3 could increase the expression of these essential antioxidant enzymes, GCL and NQO1. The results showed that CJB3 treatment led to a time-dependent enhancement of GCL and NQO1 protein expression (Figure 4). These findings demonstrate that CJB3 induces the expression of antioxidant enzymes in HepG2 cells, reinforcing its potential as a protective agent against oxidative stress.

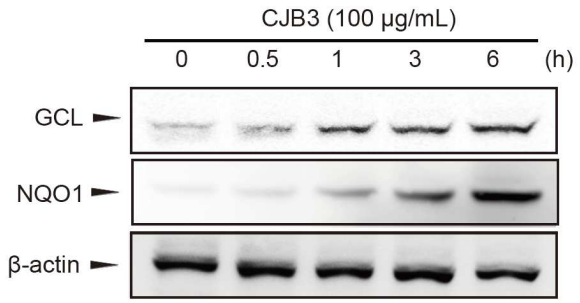


Figure 4. Effect of CJB3 on the expression of antioxidant enzymes in HepG2 cells. Cells were stimulated with CJB3 (100 μg/mL) for 0.5–6 h. The levels of marker proteins (GCL and NQO1) of antioxidant enzymes in HepG2 cell lysates as determined by immunoblotting. The experiments were repeated thrice.

3.5. CJB3 derives Nrf2 Activation in HepG2 Cell

Nrf2 serves as a master regulator of the cellular defense system, primarily focusing on enhancing the production of crucial antioxidant enzymes like GCL and NQO1[22]. To investigate the activation of Nrf2 by CJB3, we examined Nrf2 phosphorylation and its nuclear translocation. The results unequivocally showed that both Nrf2 phosphorylation and nuclear translocation increased in a time-dependent manner following CJB3 treatment (Figure 5). These findings strongly suggest that CJB3 induces Nrf2 activation in HepG2 cells, further supporting its potential as a potent inducer of cellular antioxidant defense mechanisms.

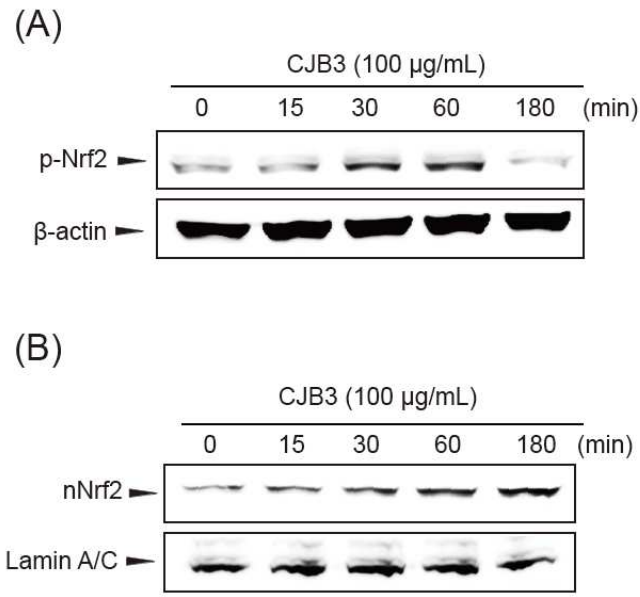


Figure 5. Effect of CJB3 on the expression of nuclear factor erythroid 2-related factor 2 (Nrf2) activation in HepG2 cells. (A) Phosphorylation of Nrf2 was measured by immunoblot analysis of lysates of HepG2 cells incubated with 100 μg/mL of CJB3 for 15 min to 180 min. Experiments were repeated three times. (B) The time-course study of nuclear translocation of Nrf2 in HepG2 cells treated with CJB3 (100 μg/mL) for 15 min to 180 min. Experiments were repeated thrice.

3.6. CJB3 induced-Nrf2 target gene via MAPK activation

Mitogen-activated protein kinase (MAPK) constitutes a family of proteins that respond to various extracellular signals, such as growth factors, cytokines, and stress[23]. These MAPK pathways, including ERK (extracellular signal-regulated kinase), JNK (c-Jun N-terminal kinase), and p38, influence the activity of Nrf2[24]. Activation of MAPK pathways can lead to the modification of Keap1, releasing Nrf2, enabling it to enter the nucleus and initiate cellular defense responses[25]. To explore whether the increased expression of Nrf2 target genes induced by CJB3 was related to MAPK activation, we conducted experiments using three MAPK inhibitors. The enhanced expressions of GCL and NQO1 (Nrf2 target genes) by CJB3 were blocked by p38 or JNK inhibitors, but not by ERK inhibitors.

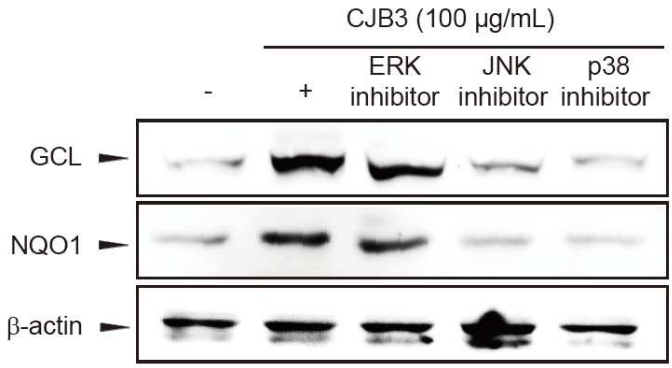


Figure 6. The effects of CJB3 on Nrf2 target gene expression via the activation of MAPK. HepG2 cells were treated with CJB3 (100 μg/mL) and/or MAPK inhibitors (ERK, p38, and JNK inhibitor; each of 10 μM) for 6 h. The experiments were repeated thrice.

3.7. CJB3 induced-Nrf2 target gene via AMPK activation

Next, we used an inhibitor (compound C) to examine whether AMPK was involved in Nrf2 activation by CJB3. AMP-activated protein kinase (AMPK) was involved in Nrf2 activation by CJB3. AMPK, often referred to as a cellular energy sensor, plays a dual role as a modulator of Nrf2 activation, not only monitoring energy status but also influencing Nrf2 activity[26]. Consequently, AMPK activation can enhance the expression of antioxidant enzymes, contributing significantly to cellular defense against oxidative stress[27]. Given this, we investigated that the induction of GCL and NQO1 (Nrf2 target genes) by CJB3 was associated with AMPK activation.

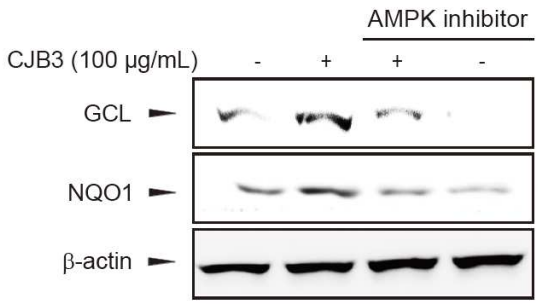


Figure 7. The effects of CJB3 on Nrf2 target gene expression via the activation of AMPK. HepG2 cells were treated with CJB3 and/or AMPK inhibitor (Compound C, 5 μM) and then immunoblotted for GCL and NQO1 proteins to determine the effects of CJB3. Cells were treated with CJB3 (100 μg/mL) in combination with 5 μM compound C for 6 h.

4. Discussion

Preserving the health of hepatocytes is essential for maintaining liver function and preventing liver injury[1]. Hepatocytes, as the primary functional cells in the liver, are responsible for a wide range of metabolic, synthetic, and detoxifying functions[3]. Any damage to these cells can result in liver dysfunction, emphasizing the critical need for hepatocyte protection in ensuring overall liver health. Nrf2 is a transcription factor that plays a central role in the cellular defense against oxidative stress. Activation of Nrf2 in hepatocytes induces the expression of antioxidant and detoxification genes, thereby protecting against liver injury[28]. Consequently, Nrf2 activation stands as a potential therapeutic target for conditions associated with oxidative stress, including specific diseases such as liver disease. In a previous study, we evaluated the antioxidant activity of fractionated CJB (70% EtOH extract), including hexane (CJB1), chloroform (CJB2), ethyl acetate (CJB3), butanol (CJB4), and water (CJB5). Our findings from the DPPH and ABTS radical-scavenging assays revealed that CJB3 exhibited the highest antioxidant activity[13]. Building on these results, our current study aimed to investigate whether CJB3 possesses a cytoprotective effect and induces antioxidant enzymes in hepatocytes. We utilized t-BHP to induce ROS production. t-BHP, a stable liquid belonging to the class of organic peroxides, is commonly used in industrial processes[19]. We confirmed that CJB3 inhibited ROS production and cell death caused by t-BHP.

The regulation of antioxidant enzymes is orchestrated by the transcription factor Nrf2, a pivotal controller of cellular responses to oxidative stress. Various upstream signaling pathways and events contribute to the release and activation of Nrf2[22]. Among these, MAPK pathways, including ERK, JNK, and p38, can modulate Nrf2 activation[29]. Additionally, AMPK activation in response to energy stress is associated with Nrf2 activation. AMPK phosphorylates and activates Nrf2, thereby influencing its stability and transcriptional activity[30]. Using their inhibitors, we verified that CJB3 increased the expression of Nrf2 target genes (GCL and NQO1) and that this was related to MAPK and AMPK activation.

The findings of the study suggest that CJB3 can prevent oxidative stress and apoptosis caused by t-BHP. In addition, it was observed that CJB3

activates JNK, p38, and AMPK which in turn enhances the expression of GCL and NQO1 (Nrf2 target genes). These results indicate that CJB3 can be a potential solution for the treatment of liver diseases.

5. Conclusions

In summary, t-BHP induces hepatocyte damage by promoting ROS production and apoptosis. However, CJB3 effectively prevented t-BHP-induced cytotoxicity by suppressing apoptosis through the reduction of ROS production, leading to increased cell viability. Furthermore, CJB3 treatment upregulated the expression of Nrf2 target genes (GCL and NQO1), through the mediation of JNK, p38, and AMPK pathways. Consequently, our findings strongly support the notion that CJB3 represents a promising candidate for treating oxidative stress-induced hepatocyte damage, highlighting its potential therapeutic significance in oxidative stress-mediated liver disease.

6. A written apology

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